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BIOLOGICAL STRUCTURE AND FUNCTION
VOLUME II

Biological Structure and Function

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Edited by

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Volume II



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Preface

In 1956 The International Union of Biological Sciences (IUBS) decided to set up a Biochemistry Section Committee, which would be a Co-ordinating Committee between IUBS and the International Union of Biochemistry (IUB) and, through a Co-ordinating Committee of IUB and the International Union of Pure and Applied Chemistry (IUPAC), would also have contact with IUPAC. It was considered that the Committee would be specifically concerned with *chemical biology* within the framework of the Unions federated to the Councils of Scientific Unions (ICSU). The members of the Biochemistry Section Committee are at present: R. Brunel (Toulouse) and O. Lindberg (Stockholm) (appointed by IUBS), M. Florkin (Liège) and T. W. Goodwin (Aberystwyth) (appointed by IUB), and P. Boyer (Minneapolis) and F. Lynen (Munich) (co-opted members). Florkin and Goodwin were elected Chairman and Secretary respectively.

The first Committee meeting was held in 1958 during the 4th International Congress of Biochemistry in Vienna. It had been visualized throughout the discussions that an important function of the Committee would be to make suggestions for various International Symposia to both IUBS and IUB. It was agreed that subjects would be appropriate only if both biochemistry and the biological sciences were combining to produce a rapidly expanding sphere of knowledge. A number of possibilities were considered at Vienna and it was eventually decided that "Biological Structure and Function" was most appropriate at this time. This idea was accepted by the two International Unions and plans began to be formulated. It was readily agreed that the most suitable centre in Europe for such a symposium was the Wenner-Gren Institute, with its well-established, international reputation in this field and, furthermore, the project had the blessing and support of Dr. Axel Wenner-Gren himself, who honoured the Symposium by agreeing to act as Patron of Honour and by attending the Inaugural Session to deliver the opening address.

The IUB and IUBS have supported this Symposium financially but the realization of the Symposium would not have been possible without the generous aid of the Wenner-Gren Foundation, and of the various bodies in different countries which support the attendance of scientists at important international meetings. It was extremely satisfying to the

organizers to know that these official bodies considered this First IUB/IUBS Joint Symposium worthy of support, and mention should be made of the National Science Foundation which supported so many of our U.S. participants; furthermore, in this connection the work done on our behalf by Dr. Elmer Stotz, the treasurer of IUB, should not be forgotten.

The organizers hope that this Symposium will be the forerunner of a long line of similar international symposia based on fruitful co-operation between biochemists and biologists from all nations.

The organizers are most grateful to the Institute of Physics, University of Stockholm, for their generosity in putting their attractive new lecture theatre at the disposal of the Symposium.

In preparing the proceedings for the press the organizers have been greatly helped by Miss J. T. Peel, who transcribed the recorded discussions, and by Mr. D. J. Howells, who prepared the subject index.

April, 1961

T. W. GOODWIN

O. LINDBERG

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MITOCHONDRIAL STRUCTURE AND FUNCTION

Effects of Thyroxine and Related Compounds on Liver Mitochondria *in Vitro**†

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I. Historical Survey

A first although not successful attempt to demonstrate an effect of thyroxine on oxidative phosphorylation in mitochondria was reported by Judah and Williams-Ashman [1] in 1951. Later the same year Judah [2] demonstrated a slight effect on the P/O ratio of liver mitochondria isolated from thyroxine-treated rats. He also compared the effect of thyroxine with that of 2,4-dinitrophenol, and pointed out that no similarity existed between the modes of action of the two compounds. At about the same time Martius and Hess [3] briefly reported that thyroxine, either administered *in vivo* or added *in vitro*, lowered the phosphorylation of isolated rat liver mitochondria. Niemeyer *et al.* [4], however, found no effect on the P/O ratio of liver mitochondria from rats treated with thyroxine *in vivo*, but were able to demonstrate a significant decrease of the respiratory control by phosphate acceptor in these mitochondria.

In the first comprehensive work on thyroxine effect *in vitro*, Lardy and Feldott [5] demonstrated in 1951 that this compound at a concentration of 10^{-5} M inhibited the oxidation of glutamate and of certain other DPN-linked substrates by a washed residue of homogenized rat kidney. The inhibition could be partly relieved by added DPN. Using particulate preparations of rat liver, a certain extent of decrease of the P/O ratio was also noticed, both when thyroxine was added *in vitro*, and in preparations from hyperthyroid animals. The following year Lardy [6] advanced the hypothesis that the hormonal effect of thyroxine resides in its capacity to uncouple one, rate-limiting, phosphorylation from the respiration, thus enhancing both respiratory rate and net output of high-energy phosphate

* This work has been supported by grants from the Swedish Medical Research Council and the Swedish Cancer Society.

† Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

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at the expense of thermodynamic efficiency. A somewhat similar hypothesis, based on a chemical relationship between thyroxine and dinitrophenol (both being substituted phenols), was also put forward by Martius [7]. In the many attempts [8-12] to prove these hypotheses experimentally, it has been possible, in some instances, to demonstrate a partial uncoupling of respiration from phosphorylation in isolated mitochondria due to thyroxine treatment, both *in vitro* and *in vivo*. However, these effects did not appear in a consistent manner, nor could a preferential uncoupling of one of the three respiratory chain phosphorylations be established.

In 1954 Hoch and Lipmann [13] reported that a consistent decrease of the P/O ratio in isolated rat liver mitochondria by thyroxine could be obtained, if the mitochondria were preincubated with thyroxine for a period of time before the addition of substrate. Hamster liver mitochondria, on the other hand, required no preincubation. However, even in this case, the results were rather inconsistent from one experiment to another. The significance of the loss of respiratory control without an actual loss of phosphorylating capacity, found in earlier work [4], was re-emphasized. In parallel papers by Bain [14] and by Mudd *et al.* [15] it was shown that the effect of thyroxine on the P/O ratio could be prevented by magnesium ions.

By this time, attention became directed towards the effect of thyroxine on mitochondrial structure. In 1953 Aebi and Abelin [16] reported that liver mitochondria from thyrotoxic rats exhibited an increased tendency to spontaneous swelling *in vitro*. Subsequently Klemperer [17] found an increased water-content in thyroxine-treated mitochondria. Tapley *et al.* [18] demonstrated in 1955 that thyroxine added *in vitro* enhances the swelling of KCl-suspended normal rat liver mitochondria. A similar effect was obtained with kidney mitochondria, while the swelling was much weaker with mitochondria from muscle, brain and testes [19]. It was also shown [20] that the P/O ratio of phosphorylating mitochondrial fragments, prepared with digitonin from liver mitochondria, was not affected by thyroxine whereas it was still sensitive to dinitrophenol. From these findings it was concluded (cf. also [21, 22]) that thyroxine, in contrast to dinitrophenol, exhibits its effect on oxidative phosphorylation by a secondary mechanism which is somehow correlated with the mitochondrial structure.

The swelling effect of thyroxine *in vitro* has been subsequently studied in great detail in a series of papers by Lehninger and associates ([23-28]; for review, cf. [29]). It emerged from these studies that this effect is similar to that obtained when mitochondria are incubated for a period of time ("aged") in a phosphate-containing medium ([30-46] for review, cf. [47]). In both cases, the swelling seems to be the result of an active process, which is typically temperature- and time-dependent. It requires the presence of an oxidizable substrate, and is prevented by respiratory

inhibitors. Moreover, it is prevented by dinitrophenol, adenine nucleotides, and in general apparently by conditions that prevent the accumulation of high-energy intermediates within the mitochondria. Significantly, active swelling is reversed by ATP, and this "contraction" of the mitochondrial structure has been shown to be reflected in an extrusion of water from the mitochondria, paralleled by a splitting of ATP. It has also been demonstrated [29, 48] that the ATP-induced contraction is dependent on the presence in the mitochondria of a specific protein fraction; the symbol, "M factor", has been used to denote this fraction.

Parallel to the swelling, the mitochondria lose their endogenous content of DPN. When DPN is added to such mitochondria in the presence of ATP, a rebinding of the DPN to the mitochondrial structure takes place. Whether the loss of DPN is a cause or a consequence of the swelling is not quite clear, although recent investigations by Kaufmann and Kaplan [49] would seem to indicate that the latter is the case.

A further characteristic feature of the process of active swelling is that it is not immediately accompanied by an uncoupling of phosphorylation from respiration. Thus, mitochondria which have reached a state of swollen structure following exposure to ageing in the presence of phosphate or thyroxine are still capable of exhibiting an electron transport-coupled phosphorylation when DPN is added to restore respiration (with succinate as substrate the situation seems to be somewhat more complicated [35], owing probably to the recently discovered requirement of high-energy phosphate for the oxidation of this substrate [50-54]). However, simultaneously with the swelling and the loss of DPN, or even preceding these effects, the mitochondria lose the tight coupling between respiration and phosphorylation, the former becoming independent of the presence of orthophosphate and ADP. In such mitochondria, thus, coupled phosphorylation *can* take place, when phosphate and phosphate acceptor are present, but respiration can proceed at maximal rate even in the absence of these additions. It has been shown by Lehninger and associates [55, 56] that this state of "loose-coupling" can be induced in intact mitochondria not only by the above treatments but also by the addition of a protein factor, called "R factor", which can be obtained from mitochondria after disruption with sonic waves; intact mitochondria thus seem to contain this factor in an inactive state. "Loose-coupling" effects can be induced in mitochondria also by a number of common uncoupling agents if these are added in low concentrations [57, 58].

Although thyroxine is not the only agent capable of enhancing active swelling and related symptoms in mitochondria—besides inorganic phosphate, calcium ions [35, 37, 59] and more recently phloridzine [60] have been shown to exhibit similar effects—several attempts have been made to explain the primary mode of action of thyroxine in terms of these

effects (cf. [29]). According to one of the visualized mechanisms, the mitochondrial DPN might constitute the target molecule for thyroxine action, the latter causing a displacement of the bound DPN and thereby a disorganization of the structure. The possibility has also been considered that thyroxine might act primarily by activating the "R factor", thus inducing a loose-coupling of phosphorylation from respiration, or alternatively by inhibiting the activation of the "M-factor" and thus interfering with the contractile mechanism responsible for the maintenance of a tight mitochondrial structure.

A marked swelling of liver mitochondria *in vivo* following treatment of rats with large doses of thyroxine has been described in electron microscopic studies by Schulz *et al.* [61]. However, when these mitochondria were isolated they exhibited normal respiration and P/O ratio, and differed from normal mitochondria only with regard to an increased susceptibility to agents eliciting swelling such as calcium ions [62].

A state of loose-coupling of the oxidative phosphorylation, of the type earlier described by Hoch and Lipmann [13] in liver mitochondria from thyrotoxic hamsters, was recently reported by Ernster *et al.* [63] to occur in skeletal muscle mitochondria from patients with thyrotoxicosis. These mitochondria revealed a markedly lowered respiratory control as compared with those from normal subjects, whereas the P/O ratio obtained in the presence of phosphate and phosphate acceptor, as well as the rate of oxidation of DPN-linked substrates, were virtually normal. Interestingly, the same findings were also made with skeletal muscle mitochondria from a patient exhibiting an extremely severe hypermetabolism (BMR around + 200%) of non-thyroid origin which is now being considered to be related to an inborn defect of the mitochondrial structure.

In summarizing this brief historical survey, it may be said that there exists today a well-established symptomatology of the action of thyroxine on isolated mitochondria *in vitro*, and that some of the symptoms, though not all, can also be seen in mitochondria exposed to toxic levels of thyroid hormone *in vivo*. However, some of these effects, such as a decreased P/O ratio, are inconsistent from one case to another, and those which are consistent, such as the enhanced swelling, the loss of bound DPN and the loose-coupling of phosphorylation from respiration, are in their nature connected with a time factor, thus giving the impression of being consequences of some other primary event.

2. Some instantaneous effects of thyroxine and related compounds on partial reactions of oxidative phosphorylation

In the present paper, certain effects of thyroxine and some related compounds on various enzyme activities in mitochondria *in vitro* will

be described, which differ from those outlined above in being both consistent and instantaneous. Furthermore, some of these effects can also be demonstrated in mitochondrial fragments and even at the level of the purified enzyme.

The reactions studied can be divided into two categories. The first category involves the mitochondrial ATPase reactions (both the dinitrophenol- and the Mg^{++} -activated ATPases), the P_i -ATP exchange reaction, and an ATP-ADP exchange reaction catalyzed by certain mitochondrial subfractions. In the second category belong certain flavin-catalyzed electron-transfer reactions, such as the DPNH diaphorase, DPNH-cytochrome *c* reductase, and the DPNH oxidase reactions, as well as a second diaphorase reaction, which is non-specific with respect to pyridine nucleotides. From the data presented the conclusion is derived that thyroxine and related compounds inhibit, in a consistent and instantaneous manner, reactions which involve a part or the whole of the flavin-linked respiratory chain phosphorylation. Some implications of these results as to the mode of action of thyroxine analogues on mitochondria *in vitro* will be discussed.

(A) ATPASE REACTIONS

Agents which uncouple oxidative phosphorylation in liver mitochondria usually also evoke an increased ATPase activity [64-68]. Two types of ATPase reactions may be distinguished. One is elicited by dinitrophenol and related uncoupling agents. This ATPase reaction occurs in structurally intact mitochondria and requires no addition of Mg^{++} to exhibit maximal activity. Another type of liver-mitochondrial ATPase appears when the structure of the mitochondria is damaged by physical or chemical means, so as to disrupt the obligatory coupling between respiration and phosphorylation. This ATPase reaction is strictly dependent on added Mg^{++} . According to a widely held opinion [65, 67, 69-78] one or both of the ATPase reactions reflect, in a modified form, a part of the reaction sequence involved in oxidative phosphorylation.

Early considerations that thyroxine and related compounds may act as uncouplers of oxidative phosphorylation were paralleled by the assumption that these agents would also evoke a high mitochondrial ATPase activity. Data presented by Lardy and Maley [10] and by Maley [79] showed that this was the case, even though the ATPase activity appearing in rat liver mitochondria in the presence of thyroxine was relatively low as compared to that induced by dinitrophenol. While recently confirming these data in our laboratory, the rather unexpected finding was made that certain thyroxine analogues markedly inhibited the ATPase activities of rat liver mitochondria, both that induced by dinitrophenol and that elicited by destruction of the mitochondrial structure.

Inhibition of dinitrophenol- and Mg^{++} -activated ATPases by thyroxine and related compounds

In Fig. 1, the effects of thyroxine, triiodothyronine and desaminothyroxine on the dinitrophenol induced ATPase of rat liver mitochondria are illustrated. Of the three compounds, desaminothyroxine exhibited the strongest inhibition, giving half-inhibition at a concentration of about 0.02 mM. The effect of the same compounds on the Mg^{++} -activated ATPase is shown in Fig. 2. For the study of this reaction a preparation of mitochondrial fragments, obtained after disruption of mitochondria with a rapidly rotating Super-Thurrax blender was used.

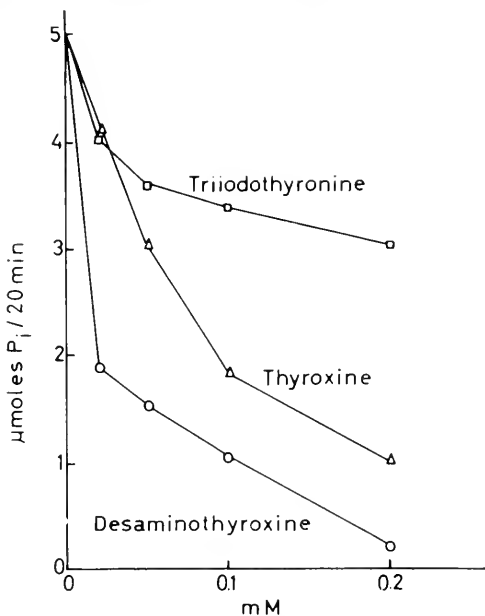


FIG. 1. Effect of L-thyroxine, DL-triiodothyronine and desaminothyroxine on the dinitrophenol-induced ATPase activity of rat liver mitochondria. For experimental details see [76].

The procedure was adapted from Kielley and Kielley [80] who devised it for enriching mitochondrial ATPase free from adenylate kinase. As can be seen in Fig. 2, the ATPase activity of the Kielley and Kielley preparation was also inhibited by the three compounds tested, and again, desaminothyroxine exhibited the strongest inhibition. The half inhibitory concentration of desaminothyroxine was roughly the same as in the case of the dinitrophenol-induced ATPase. However, in contrast to this latter reaction, the inhibitions given by the triiodothyronine and thyroxine were not progressive with concentration, but levelled off at about 0.1 mM to

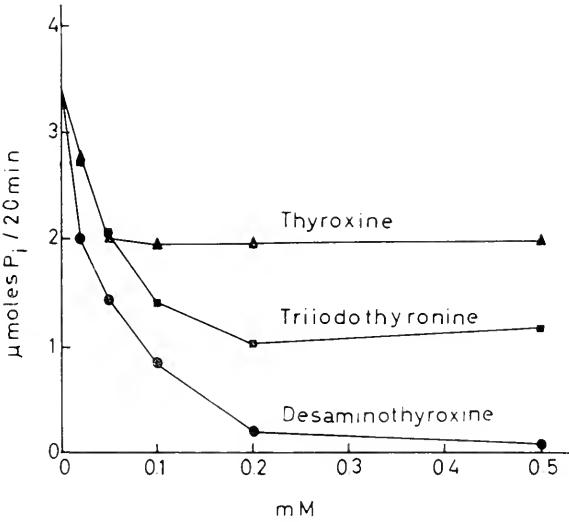


FIG. 2. Effect of L-thyroxine, DL-triiodothyronine and desaminothyroxine on the ATPase activity of mitochondrial fragments prepared according to Kielley and Kielley [80]. For experimental details see [84].

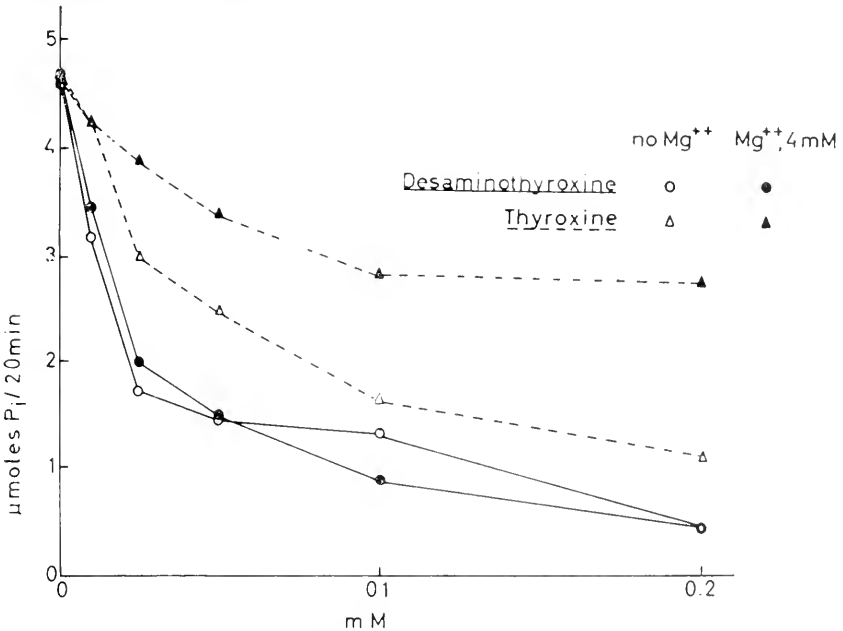


FIG. 3. Effect of added Mg⁺⁺ on the sensitivity of the dinitrophenol-induced ATPase activity of rat liver mitochondria to DL-thyroxine and desaminothyroxine. When indicated, Mg⁺⁺ was added in a final concentration of 4 mM. Other experimental conditions as in Fig. 1.

give a maximal inhibition of about 30 and 60%, respectively. A similar pattern of inhibition could be obtained also in the case of the dinitrophenol-induced ATPase if this reaction was measured in the presence of added Mg^{++} (Fig. 3). Thus, whereas added Mg^{++} did not alter the inhibition of the dinitrophenol-induced ATPase by desaminothyroxine, it rendered the inhibition with thyroxine less efficient and with a maximal inhibition of about only 30%. It would seem that this effect of Mg^{++} was not due

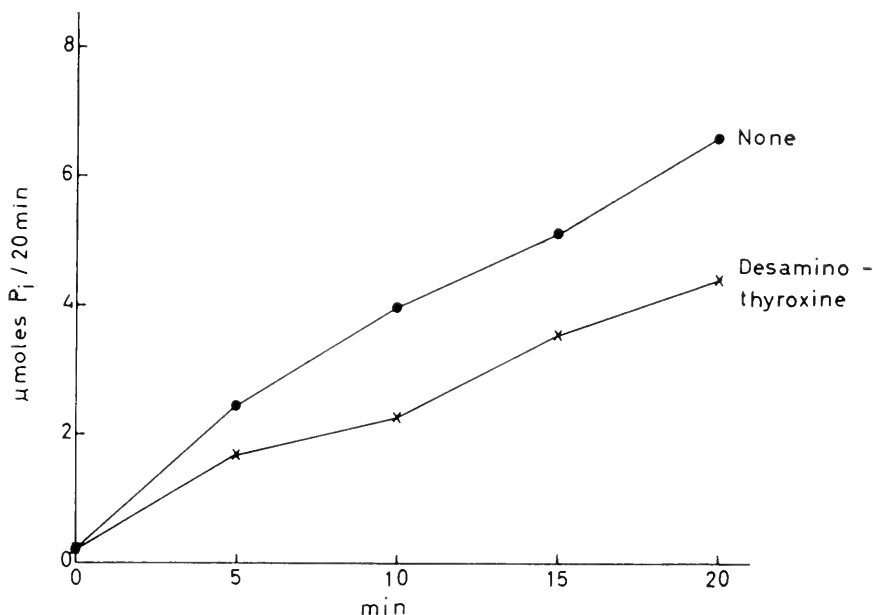


FIG. 4. Time-course of inhibition of the dinitrophenol-induced ATPase reaction by desaminothyroxine. When indicated desaminothyroxine was added in a final concentration of 0.2 mM. Other experimental conditions as in Fig. 1.

primarily to a binding of thyroxine (in which case the protection by Mg^{++} should have been overcome with higher concentrations of thyroxine), but rather to an ability of Mg^{++} to restrict the number of active sites in the preparation accessible to thyroxine. For this reason the investigations to follow were performed with desaminothyroxine.

The inhibitory effect of desaminothyroxine on the dinitrophenol-induced ATPase reaction was instantaneous, as shown in Fig. 4. Pre-incubation with desaminothyroxine prior to the addition of ATP did not influence the extent of inhibition, neither in the case of this reaction, nor in the case of the Mg^{++} -activated ATPase reaction catalyzed by mitochondrial fragments (Fig. 5).

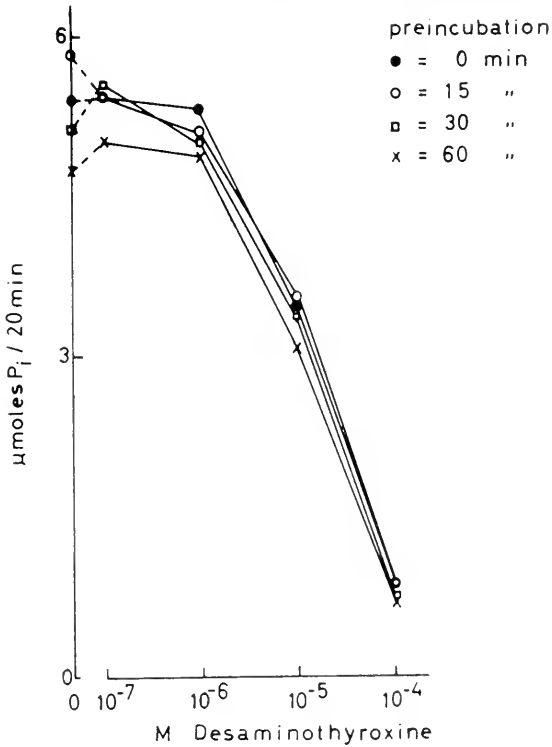


FIG. 5. Lacking effect of preincubation on the desaminothyroxine sensitivity of the ATPase activity of mitochondrial fragments prepared according to Kielley and Kielley [80]. Experimental conditions as in Fig. 2.

Comparison with other ATPase inhibitors

A further characterization of the effect of desaminothyroxine on the mitochondrial ATPase reactions was considered possible by comparing it with the effects of known ATPase inhibitors.

It has been known for some time that the liver mitochondrial ATPase reactions are inhibited by azide [75, 81-83] and by a number of flavin antagonists, including atebtrin [76] and chlorpromazine [77]. The effect of the flavin antagonists on the dinitrophenol-induced ATPase is diphasic, consisting of a stimulation at low concentrations and an inhibition at high concentrations [76, 77]. Figure 6 compares the effects of desaminothyroxine, azide, atebtrin and chlorpromazine on the dinitrophenol-induced ATPase activity on the basis of concentration. Desaminothyroxine was the most potent of the four inhibitors, and its effect lacked the dual character shown by the flavin antagonists.

It was shown previously that amytal inhibits slightly the dinitrophenol-induced ATPase [75], and that this inhibition can be greatly potentiated if a stimulating concentration of atebtrin [76] or chlorpromazine [77] is added. Fig. 7 illustrates this effect and shows that a similar potentiation did not occur with azide or desaminothyroxine. In fact, desaminothyroxine seemed even to eliminate the slight inhibition given by amytal.

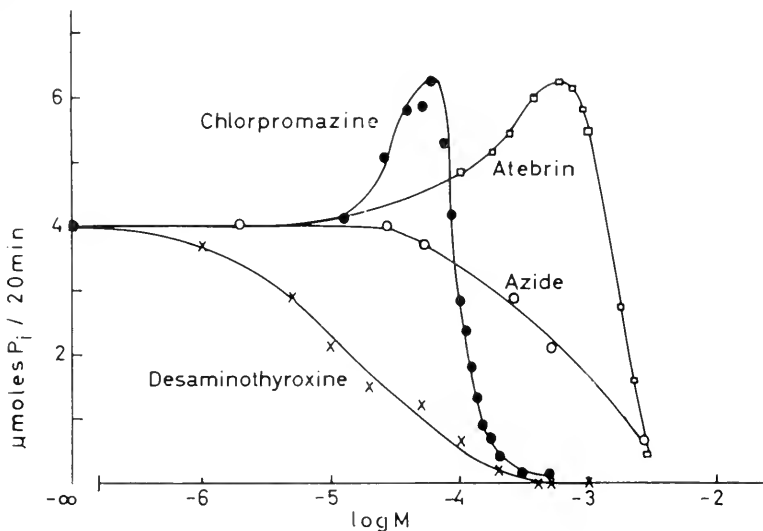


FIG. 6. Comparison of effects of azide, atebtrin, chlorpromazine and desaminothyroxine on the dinitrophenol-induced ATPase activity of rat liver mitochondria. Experimental conditions as in Fig. 1.

An interesting effect of atebtrin was discovered by observing that this compound in a concentration of 0.5 mM was able to relieve almost completely the inhibitory effect of desaminothyroxine on the dinitrophenol-induced ATPase reaction (Table I). Peculiarly enough, this effect of atebtrin was not shared by chlorpromazine. Similarly, no atebtrin-like effect was found with flavin nucleotides.

It appeared from the above findings that the effect of desaminothyroxine on the dinitrophenol-induced ATPase clearly differed from those of the flavin antagonists, whereas the difference from that of azide was less obvious. However, a clear-cut distinction was found also between desaminothyroxine and azide in the effects of the inhibitors on the Mg^{++} -activated ATPase of the Kielley and Kielley preparation. As was reported previously [76], the Mg^{++} -activated ATPase is characterized by a stimulation, up to about 50%, by 0.5–1 mM sodium dithionite. This compound, however, not only stimulates the Mg^{++} -activated ATPase reaction but

TABLE I

ABOLITION OF THE DESAMINOTHYROXINE-INHIBITION OF THE DINITROPHENOL-INDUCED ATPase ACTIVITY OF LIVER MITOCHONDRIA BY ATEBRIN

Experimental conditions as in Fig. 1.

Desamino- thyroxine, M	moles P_i 20 min.				
	None	Atebrin, 5×10^{-4} M	Chlorpro- mazine, 5×10^{-5} M	Chlorpro- mazine, 10^{-4} M	FAD, 5×10^{-4} M
0	4.2	5.9	6.4	3.7	4.0
2×10^{-5}	1.3	4.7	1.8	1.1	1.6
2×10^{-4}	0.2	4.0	0.5	0.9	0.2

is also able to counteract to a remarkable extent the inhibitory effects of azide, atebtrin and chlorpromazine in this reaction. This latter effect of dithionite, which is illustrated in Fig. 8, was strongest in the case of azide,

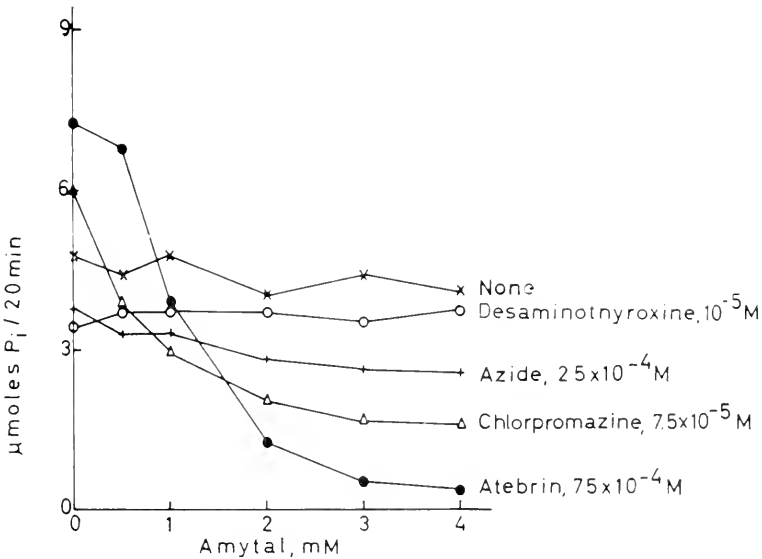


FIG. 7. Influence of azide, atebtrin, chlorpromazine and desaminothyroxine on the amytal sensitivity of the dinitrophenol-induced ATPase activity of rat liver mitochondria. Experimental conditions as in Fig. 1.

followed by atebtrin and chlorpromazine. In the case of the inhibition by desaminothyroxine no counteraction by dithionite could be observed. The only other inhibitor of the Mg^{++} -activated ATPase which was found not to respond to dithionite was pentachlorophenol.

Finally, as briefly reported elsewhere [84], desaminothyroxine exhibited

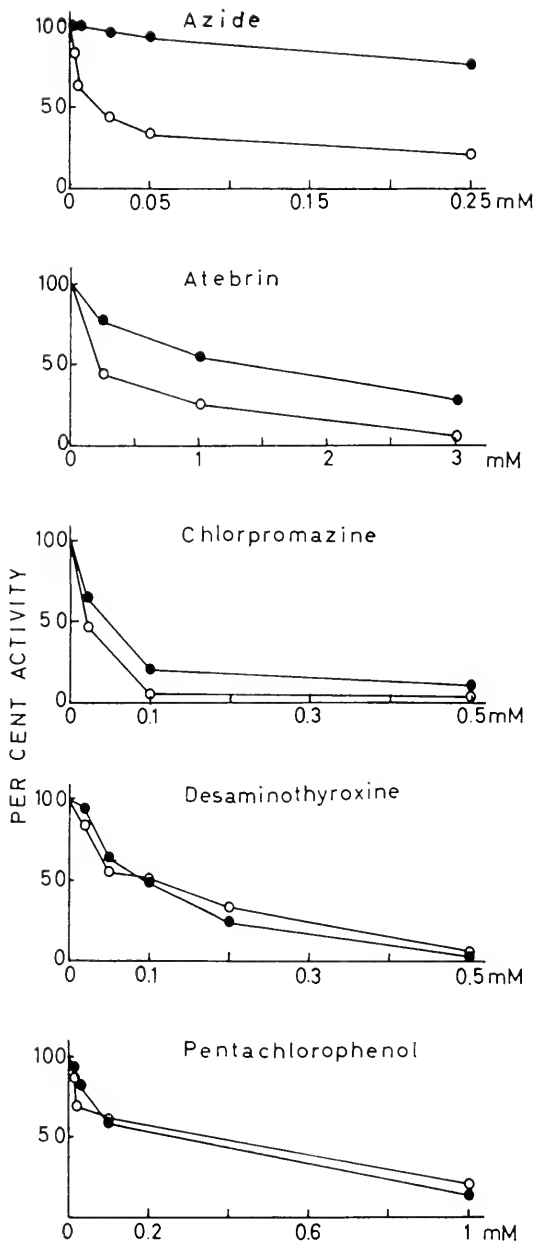


FIG. 8. Influence of sodium dithionite on the inhibition of the ATPase activity of mitochondrial fragments by various agents. Experimental conditions as in Fig. 2. The ATPase activity of the sample without inhibitors was 5.11 and $7.03 \mu\text{moles P}_i/20 \text{ min.}$ in the absence $\text{---}\circ$ and presence $\text{---}\bullet$ of 0.5 mM dithionite, respectively.

no photosensitizing effect on the Mg^{++} -activated ATPase, in contrast to the flavin antagonists, atebtrin and chlorpromazine.

From the results presented above it would thus appear that the effect of desaminothyroxine, and probably of thyroxine analogues in general, differs in its mechanism from those of other known inhibitors of the two types of liver mitochondrial ATPase reactions.

(B) EXCHANGE REACTIONS

P_i-ATP exchange

The effect of desaminothyroxine on the P_i -ATP exchange reaction of liver mitochondria was investigated using the conditions previously established in this laboratory [85]. Under these conditions the ATP and P_i concentration are virtually constant during the measurement, and the exchange rate is about $0.35 \mu\text{mole}/\text{min.}$ per mitochondria from 200 mg. wet-weight liver. As shown in Table II, the P_i -ATP exchange reaction

TABLE II

INHIBITION OF THE P_i -ATP EXCHANGE REACTION OF RAT LIVER MITOCHONDRIA BY DESAMINOTHYROXINE

Assay conditions as in [85], except that 50, rather than 20, $\mu\text{moles } P_i$ were added per sample.

Desamino- thyroxine M	Per cent ^{32}P in ATP after	
	3 min.	13 mins.
0	3.91	12.05
10^{-6}	3.92	11.30
10^{-5}	1.89	2.74
10^{-4}	1.25	0.72

was strongly inhibited by desaminothyroxine, 10^{-5} M giving an almost complete inhibition. Again, the inhibition was present from the onset of the incubation, the preincubation with desaminothyroxine prior to the addition of P_i and ATP had no influence on its extent.

ATP-ADP exchange

Wadkins and Lehninger [86] described recently the occurrence of an exchange reaction between ATP and ADP in phosphorylating digitonin-preparations. This reaction was characterized by a sensitivity to dinitrophenol which was lost, without loss of the exchange activity, when the preparations were damaged so as to lose their phosphorylating capacity. Azide, also, although not inhibitory to the ATP-ADP exchange reaction,

was able to render the reaction insensitive to dinitrophenol. Hence, Wadkins and Lehninger [86] proposed that the dinitrophenol-insensitive ATP-ADP exchange reaction represents the terminal step of phosphate transfer in respiratory chain phosphorylation. Since a similar conclusion concerning the Mg^{++} -activated ATPase was previously reached in our laboratory [75-78] it was of interest to investigate whether the ATPase activity of the preparation of mitochondrial fragments was paralleled by an ATP-ADP exchange. Such a connection between the two reactions has recently been postulated by Bronk and Kielley [87] from data obtained with phosphorylating fragments of sonicated mitochondria. If such a connection existed, it was of interest to investigate whether the ATP-ADP exchange reaction was also sensitive to desaminothyroxine.

TABLE III

INFLUENCE OF SOME AGENTS ON THE ATPASE AND ATP-ADP EXCHANGE REACTIONS IN MITOCHONDRIAL FRAGMENTS PREPARED ACCORDING TO KIELLEY AND KIELLEY [80].

Conditions: for ATP-ADP exchange see [87] and for ATPase see exp. in Fig. 2. Incubation for 4 min. at 30°.

Additions	μ mole P transferred	
	ATPase	ADP exchange
(1) none	0.79	0.60
10 ⁻⁴ M azide	0.17	0.63
10 ⁻⁴ M atebtrin	0.42	0.64
10 ⁻³ M AMP	0.73	0.31
(2) none	0.61	0.45
2 × 10 ⁻² M NaF	0.08	0.34
2 × 10 ⁻³ M AMP	0.57	0.14
2 × 10 ⁻³ M AMP + 2 × 10 ⁻² M NaF	0.08	0.07

The ATP-ADP exchange was measured by using terminally labelled ³²P-ADP following the procedure described by Bronk and Kielley [87]. Table III summarizes some properties of the mitochondrial fragment preparation regarding ATPase and ATP-ADP exchange activities. In accordance with the findings of Wadkins and Lehninger [86] the exchange reaction was not inhibited by a concentration of azide which strongly inhibited the ATPase. A similar effect was obtained with sodium fluoride. Conversely, however, AMP at a concentration of 2 × 10⁻³ M strongly inhibited the exchange reaction but left the ATPase activity practically unaffected.

In Table IV the effect of desaminothyroxine on the ATP-ADP exchange reaction is shown. The exchange was inhibited almost completely

by 2×10^{-4} M desaminothyroxine. The extent of inhibition was thus comparable to that of the ATPase activity. It was found on the other hand (Table IV) that an ATP-ADP exchange reaction occurred also in the supernatant obtained in this preparation, and that also this reaction was strongly inhibited by desaminothyroxine. This fraction was free of ATPase activity, in agreement with Kielley and Kielley [80]. The exchange activity found in the supernatant was actually higher than that of the sediment. It was distinct from the latter as indicated by the fact that the residual activity could not be removed from the pellet by washing. The possibility was considered that the ATP-ADP exchange activity of the supernatant might be a reflection of the adenylate kinase reaction, which is recovered in this fraction in the present procedure. However, the following findings indicated that the two activities were not correlated: (1) The

TABLE IV

EFFECT OF DESAMINOTHYROXINE ON THE ATP-ADP EXCHANGE AND ATPASE REACTIONS OF SUBMITOCHONDRIAL FRACTIONS PREPARED ACCORDING TO KIELLEY AND KIELLEY [80].

Conditions as in Table III, except that time of incubation was 2 min. Sediment and supernatant assayed in equivalent amounts in terms of wet weight liver.

Preparation	Additions	ATPase activity μ moles P hydrolyzed	Exchange activity μ moles P exchanged
Sediment	none	0.33	0.56
	desaminothyroxine, 0.2 mM	0.11	0.06
Supernatant	none	0.00	1.46
	desaminothyroxine, 0.2 mM	0.00	0.21

adenylate kinase reaction (as measured with ADP as substrate and hexokinase and glucose as trapping agent for the ATP) was unaffected by 2×10^{-4} M desaminothyroxine whereas the ATP-ADP exchange was almost completely inhibited (cf. Table IV). (2) The adenylate kinase reaction is inhibited by 2×10^{-2} M sodium fluoride [88], whereas the ATP-ADP exchange was virtually unaffected (cf. Table III). (3) The ATP-ADP exchange activity of the supernatant compared with the net adenylate kinase activity of the same fraction was considerably higher than the corresponding ratio of the two activities in a purified preparation of muscle myokinase.

It would seem from these data that a desaminothyroxine-sensitive ADP-ATP exchange reaction is present in subfractions of rat liver mitochondria; however, the relation of this reaction to the ATPase is not clear, since it is present both in the fraction in which the ATPase is concentrated and in the fraction devoid of ATPase activity.

(c) ATP-SPLITTING REACTIONS OF NON-MITOCHONDRIAL ORIGIN

At this stage it was of interest to test the effect of desaminothyroxine on ATP-splitting reactions of non-mitochondrial origin. Myosin ATPase, muscle myokinase, potato apyrase, and yeast hexokinase, were all unaffected by a concentration of desaminothyroxine of 10^{-4} M (Table V), indicating that desaminothyroxine is not a general inhibitor of ATP-splitting enzymes. A similar correlation was previously [89] reached concerning atebirin and chlorpromazine. It may be of interest on the other hand that a liver microsomal ATPase recently studied in our laboratory [90] seems to be sensitive both to atebirin and chlorpromazine and to thyroxine analogues.

TABLE V

EFFECT OF DESAMINOTHYROXINE ON A NUMBER OF ATP-SPLITTING ENZYMES

The mitochondrial ATPase, myosin, potato apyrase, and hexokinase were assayed in the manner described by Löw [89]. Myokinase was assayed by measuring the decrease in 7 min.-P in the presence of enzyme, hexokinase and glucose.

Enzyme	μ moles ATP split			
	0	10^{-6}	10^{-5}	10^{-4}
	M desaminothyroxine			
Submitochondrial ATPase	5.1	5.0	3.2	0.9
Myosin-ATPase	5.0	5.0	5.0	4.5
Potato Apyrase	5.8	5.7	5.7	5.2
Hexokinase	6.1	6.0	5.9	4.6
Myokinase	4.1	4.9	4.7	4.8

(d) DIAPHORASE REACTIONS

Previous work in this laboratory [76-78, 85] has given rise to the concept that the mitochondrial ATPase reactions, both that induced by dinitrophenol in intact phosphorylating mitochondria, and the Mg^{++} -activated ATPase reaction appearing in structurally damaged mitochondrial preparations, involve the diaphorase flavoprotein as intermediate phosphate carrier. A possible explanation for the sensitivity of these reactions to thyroxine and related compounds would seem therefore to be that these compounds interfere in some way with the mitochondrial diaphorase. The Kielley and Kielley preparation proved to be a suitable system for investigating this question, since it was found [53] that this preparation exhibited besides a high Mg^{++} -activated ATPase activity a DPNH diaphorase reaction also. It was found, moreover, that the diaphorase present in this preparation was an integral part of a mitochondrial DPNH oxidase system, as indicated by its sensitivity to both amytal and

antimycin A. A further valuable property of this preparation was that it also contained the external type of DPNH-cytochrome *c* reductase, known to occur in liver mitochondria and characterized by an insensitivity to amyltal and antimycin A [38, 39, 47, 91-95].

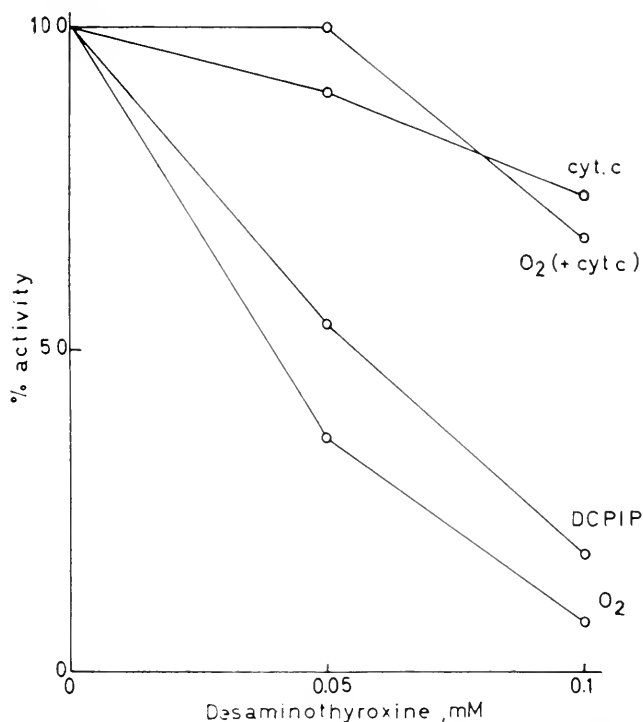


FIG. 9. Effect of desaminothyroxine on the oxidation of DPNH by various electron acceptors in mitochondrial fragments prepared according to Kielley and Kielley [80]. All assay systems contained 0.02 M phosphate buffer, pH 7.5, and 0.1 mM DPNH, in a final volume of 3 ml. In the case of O₂ as acceptor, either no further additions were made (line marked "O₂"), or 0.005 mM cytochrome *c* was added ("O₂ + cyt. *c*"), and the oxidation of DPNH was followed at 340 m μ . In the case of 2,6-dichlorophenolindophenol ("DCPiP") as terminal electron acceptor, the dyestuff was added in a final concentration of 0.04 mM, and its reduction was followed at 600 m μ ; in the case of cytochrome *c* ("cyt. *c*") this was added in a final concentration of 0.05 mM, and its reduction was followed at 550 m μ . In both latter cases, 0.33 mM KCN was included in the test. "100% activity" was (in terms of μ moles DPNH oxidized min. per g. liver): 0.146 with O₂, 0.218 with O₂ + cyt. *c*, 0.620 with cyt. *c*, and 0.487 with DCPiP as electron acceptor.

As can be seen in Fig. 9, desaminothyroxine greatly inhibited the DPNH oxidase activity of the Kielley and Kielley preparation as measured without added cytochrome *c*, as well as the diaphorase activity as measured

with 2,6-dichlorophenolindophenol as the terminal electron acceptor. At the same time desaminothyroxine only slightly inhibited the DPNH oxidase activity obtained in the presence of a catalytic amount of cytochrome *c* and the DPNH-cytochrome *c* reductase activity as measured with cytochrome *c* as terminal electron acceptor. As shown in Table VI, the sensitivity to desaminothyroxine of the diaphorase reaction was roughly equal to that of the Mg^{++} -activated ATPase and that the desaminothyroxine sensitivity of the latter reaction was not influenced by the presence of DPNH and cytochrome *c*. Conversely, addition of ATP and Mg^{++} to the DPNH-cytochrome *c* reductase system did not increase the sensitivity of this system to desaminothyroxine.

TABLE VI

COMPARISON OF EFFECTS OF DESAMINOTHYROXINE ON DPNH DIAPHORASE, DPNH-CYTOCHROME *c* REDUCTASE AND ATPASE ACTIVITIES OF MITOCHONDRIAL FRAGMENTS PREPARED ACCORDING TO KIELLEY AND KIELLEY [80].

For experimental conditions see Figs. 2 and 9.

Reaction	% inhibition by 10^{-4} M desaminothyroxine
DPNH diaphorase (in presence of ATP and Mg^{++})	81
DPNH-cyt. <i>c</i> red. (in absence of ATP and Mg^{++})	18
DPNH-cyt. <i>c</i> red. (in presence of ATP and Mg^{++})	24
ATPase (in absence of DPNH and cyt. <i>c</i>)	84
ATPase (in presence of DPNH and cyt. <i>c</i>)	76

It would appear to follow from these data that the DPNH diaphorase component of the amytal- and antimycin A-sensitive mitochondrial DPNH oxidase, which probably represents the main phosphorylative pathway of terminal electron transport in the intact liver mitochondria, is inhibited by desaminothyroxine to the same extent as the mitochondrial ATPase reactions. In contrast, the non-phosphorylating amytal- and antimycin A-insensitive DPNH-cytochrome *c* reductase appears to be much less sensitive to this agent.

Another pyridine nucleotide oxidizing flavoprotein which shows a relatively high sensitivity to thyroxine analogues is the so-called DT diaphorase. This enzyme, the detection [96, 97] and purification [98] of which was reported some time ago, and which now [53] appears to be identical with the vitamin K reductase of Martius and collaborators [99-102], catalyzes the oxidation of both DPNH and TPNH by various dyestuffs and quinones. The enzyme occurs mainly in the soluble cytoplasm but is present to a small extent also in mitochondria and microsomes

[98, 103]. The inhibition of this enzyme by thyroxine and related compounds is illustrated by Fig. 10. The half inhibitory concentration lies at around 2×10^{-5} M in the case of desaminothyroxine and 6×10^{-5} M in the cases of thyroxine and triiodothyronine.

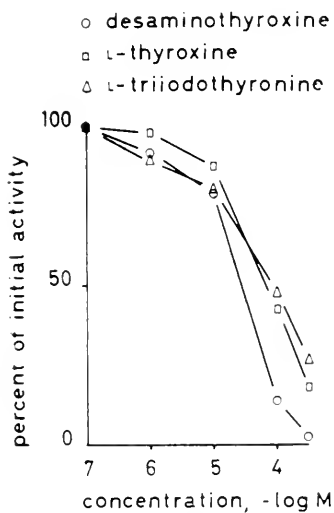


FIG. 10. Effect of L-thyroxine, L-triiodothyronine and desaminothyroxine on DT-diaphorase. The 450-fold purified enzyme was prepared and assayed as described in [98].

(E) RESPIRATION AND PHOSPHORYLATION

All the effects of thyroxine and related compounds described up to now in this paper were consistent in their occurrence and magnitude from one experiment to another. They were also all instantaneous effects, the extent of inhibition being independent of the time of measurement. In sharp contrast, no consistency was obtained when the effects of the same compounds on the respiration and phosphorylation were investigated. Furthermore the effects were often progressive with the time of incubation. To illustrate this inconsistency, some of the experiments performed are summarized in Table VII. It can be seen that both thyroxine and desaminothyroxine were able to inhibit respiration (measured with glutamate as substrate) in some experiments (Expts. 2a, 4a, 6a, 1b, 2b, 3b, 4b, 5b), whereas in others, virtually no respiratory inhibition was obtained at a concentration as high as 10^{-4} M for both compounds (Expts. 1a, 6b). In general desaminothyroxine was more inhibitory than thyroxine, although reservation must be made here for the presence of magnesium ions in the system. In no case was there a clear-cut uncoupling effect observed, the P/O

TABLE VII

EFFECT OF L-THYROXINE AND DESAMINOTHYROXINE ON RESPIRATION AND PHOSPHORYLATION OF RAT LIVER MITOCHONDRIA IN ABSENCE AND PRESENCE OF ADDED DPN.

Each Warburg-flask contained: 10 mM L-glutamate, 25 mM potassium phosphate, pH 7.5, 1 mM ATP, 4 mM MgCl₂, 125 mM sucrose, 30 mM glucose, an excess of yeast hexokinase, and, when indicated, 0.05 mM DPN, in a final volume of 2 ml. Gas phase, air. Centre well: 0.2 ml 2 M KOH. Temp., 30°. Time of incubation, 20 min.

(a) *L-Thyroxine*

Expt. No.	Amount of mitochondria per flask (mg. eq. liver)	L-Thyroxine mM	Without DPN			With DPN		
			Oxygen, μ atoms	Phosphate, μ moles	P/O	Oxygen, μ atoms	Phosphate, μ moles	P/O
1a	200	0	9.7	25.3	2.62	11.1	24.0	2.16
		0.1	9.5	24.0	2.50	9.8	3.3	0.33
2a	200	0	5.8	15.5	2.67	6.0	15.6	2.58
		0.05	0.6	1.7	(2.91)	5.5	1.2	0.21
	300	0	9.2	22.4	2.43	9.2	23.7	2.50
		0.05	6.0	14.3	2.40	8.9	2.8	0.31
400	0	13.4	32.9	2.40	14.5	33.0	2.28	
	0.05	11.9	29.8	2.50	13.2	6.5	0.49	
3a	300	0	13.0	34.4	2.65	13.1	34.7	2.65
		0.05	10.0	23.3	2.33	10.8	25.8	2.40
4a	200	0	9.5	23.2	2.44	11.1	22.8	2.06
		0.04	7.9	16.3	2.06	7.2	18.0	2.52
		0.05	4.4	9.8	2.22	7.6	6.9	0.91
5a	200	0	8.0	21.3	2.66	8.7	20.3	2.33
		0.05	7.3	19.6	2.69	9.1	18.9	2.08
		0.05	7.8	16.8	2.15	9.2	11.4	1.24
6a	400	0	12.5	35.4	2.83	12.8	31.3	2.45
		0.05	8.8	24.5	2.79	11.1	9.9	0.89
		0.1	4.6	10.8	2.35	12.8	3.6	0.28
7a	200	0	9.6	27.2	2.83	10.3	27.0	2.62
		0.05	7.5	21.6	2.86	9.5	11.0	1.16
	300	0	14.3	40.3	2.82	14.6	40.5	2.78
		0.05	12.7	35.3	2.78	13.2	34.9	2.64

TABLE VII—*continued**(b) Desaminothyroxine*

Expt. No.	Amount of mitochondria per flask (mg. eq. liver)	Desaminothyroxine, mM	Without DPN			With DPN		
			Oxygen μ atoms	Phosphate μ moles	P O	Oxygen μ atoms	Phosphate μ moles	P O
1b	200	0	9.7	25.3	2.62	11.1	24.0	2.16
		0.1	0	1.4	—	5.4	2.5	0.46
2b	300	0	18.3	45.7	2.50	18.5	45.0	2.44
		0.03	16.0	43.6	2.72	16.7	42.1	2.52
		0.1	9.5	14.4	1.52	16.6	40.6	2.45
3b	200	0	7.5	19.9	2.64	6.3	16.2	2.56
		0.05	0.9	1.4	(1.49)	5.6	3.7	0.66
		0.1	0.2	—	—	2.7	—	—
4b	200	0	11.3	31.6	2.81	12.3	32.3	2.63
		0.02	10.1	26.9	2.66	10.2	27.0	2.66
		0.05	4.6	8.4	1.85	5.3	6.6	1.25
5b	200	0	7.7	18.1	2.36	9.3	19.6	2.11
		0.03	6.3	16.2	2.57	9.5	20.9	2.20
		0.1	2.2	1.0	0.45	7.8	1.6	0.21
6b	300	0	13.0	38.0	2.92	13.8	34.0	2.46
		0.03	11.8	31.9	2.70	12.3	33.0	2.68
		0.1	10.2	24.8	2.43	11.7	28.9	2.47

ratios being roughly normal even in those cases where respiration was partially inhibited. Added DPN was able to restore the inhibited respiration and the restoration was as a rule complete in the case of thyroxine but most often only partial in the case of desaminothyroxine. In the case of desaminothyroxine inhibition, the stimulation of the respiratory rate by DPN was accompanied either by no change or by an increase in the rate of phosphate uptake. Most peculiarly, a somewhat different effect of DPN on phosphorylation was obtained in the presence of thyroxine. In this case the increased respiratory rate was never followed by an increase in phosphate uptake and often it even resulted in a serious decrease of the latter, thus giving the impression of a true uncoupling effect (Expts. 1a, 2a, 4a, 5a, 6a). Despite great efforts it has not yet been possible to obtain this effect in

a consistent manner and it would appear that it occurs only in a very narrow range of thyroxine/mitochondrial protein ratio (cf. Expt. 2a).

Thus the situation especially as far as thyroxine is concerned seems to be very complicated indeed. This is further emphasized by the recent findings of Bronk [104] and of Dallam *et al.* [105, 106] that in their systems thyroxine was even able to cause an increase of the phosphate uptake coupled to the oxidation of β -hydroxybutyrate.

3. Concluding remarks

Evidence has been presented above that thyroxine analogues inhibit in a consistent and instantaneous manner the P_i -ATP exchange and dinitrophenol-induced ATPase reactions taking place in intact liver mitochondria, as well as the Mg^{++} -activated ATPase and ATP-ADP exchange reactions observed in mitochondrial fragments. A common denominator of all these reactions is that they are considered to include one or several steps of the reaction sequence involved in phosphorylation coupled to electron transport. The succinate-linked reduction of mitochondrial DPN, a process which is also considered to involve a partial reaction of electron-transport-coupled phosphorylation, has recently been reported by Chance and Hollunger [107] to be highly sensitive to thyroxine.

It has been concluded from previous work in this laboratory [76-78, 85] that the mitochondrial P_i -ATP exchange and ATPase reactions reflect predominantly only one of the three phosphorylations occurring along the respiratory chain, that located in the DPN-flavin region. Also the succinate-linked reduction of mitochondrial DPN is thought to involve primarily a partial reversal of this phosphorylation [51, 53, 107, 108]. It would therefore seem that the observed instantaneous effects of thyroxine and related compounds concern primarily the DPN-flavin-coupled phosphorylation. The finding that these compounds inhibited the diaphorase component of the amytal- and antimycin A-sensitive DPNH oxidase system, is consistent with this conclusion, and may indicate that the effect of thyroxine and related compounds on the flavin-linked phosphorylation consists of a direct action on this enzyme. The DPNH-cytochrome *c* reductase, which is insensitive to amytal and antimycin A, and which probably represents a non-phosphorylating pathway of electron transport [38, 39, 47, 91-95], was only marginally inhibited by the compounds studied. The significance of the observed inhibition of the DT diaphorase cannot be understood as yet, since the role of this enzyme in mitochondria is unclear (cf. [109-112]).

The effect of thyroxine and related compounds was clearly less reproducible on the integrated processes of respiration and phosphorylation than it was when studied with the above component reactions as test

systems. This lack of reproducibility may perhaps be explained by assuming that the flavin-linked electron transport and phosphorylation reactions usually occur in the mitochondria at an excess capacity in comparison to the overall rates of respiration and phosphorylation. Such an assumption would be in line with the repeated findings [69, 83, 113-115] that the rate of P_i -ATP exchange considerably exceeds the rate of phosphate uptake in mitochondria under conditions of maximal respiration and phosphorylation. The possibility that the inconsistent and gradual character of the effects of thyroxine and related compounds on respiration and phosphorylation could be due to a poor penetration of these compounds through the intact mitochondrial membrane seems improbable, since consistent and instantaneous effects ensued in the case of the P_i -ATP exchange and dinitrophenol-induced ATPase reactions, both of which were measured in intact mitochondria. Moreover, as has been demonstrated recently by Tapley and Basso [116], the uptake of thyroxine and related compounds by mitochondria occurs in an instantaneous manner.

As outlined in the introduction, mitochondrial swelling and related symptoms seem to be dependent on an active oxidative phosphorylation; this is indicated by the findings that swelling does not occur in the absence of oxidizable substrate, and is prevented by respiratory inhibitors and by dinitrophenol. This state of affairs raises the question as to how mitochondria in a state of active phosphorylation are able to maintain their structural integrity. It has been pointed out [29] that the ATP-induced contraction of the mitochondria cannot be due to a simple reversal of the process underlying the swelling, since the contraction is not inhibited by dinitrophenol. However, there are now indications [50-53, 85, 117, 118] that dinitrophenol interferes only with the forward reaction, and not the reversal, of electron transport-coupled phosphorylation. It has also been shown [26] that amytal inhibits the ATP-induced contraction of mitochondria and that this effect is not shared by antinycin A and cyanide. These facts point thus to the possibility that a reversal of the flavin-linked phosphorylation may play a part in the contraction of the mitochondria. It would not seem inconceivable, therefore, that the great excess capacity of this phosphorylation in the mitochondria as compared with the overall rate of respiration and phosphorylation might be endowed with the important function of maintaining the actively phosphorylating mitochondrion in a structurally and functionally intact shape. It would be understandable, then, that exposure of mitochondria to toxic concentrations of thyroid hormone, thereby depriving them of this excess capacity of the flavin-linked phosphorylation, may lead to a gradual loss of their integrated properties.

In summary, then, the present data seem to provide a first information about a direct effect of thyroxine and related compounds on the mito-

chondrial oxidative phosphorylation system, with the flavoenzyme component of the respiratory chain as the probable site of action. The relation of this effect to those established in previous literature, such as loss of respiratory control, release of bound DPN, enhanced swelling of the mitochondrial structure, and general uncoupling of phosphorylation from respiration, remains for the moment unclear. It is tempting to speculate, however, that, since the latter effects are all time-dependent whereas those described in the present paper are instantaneous, there might exist a cause-effect relationship between them.

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Discussion

HESS: I would like to mention earlier experiments regarding the protecting activity of thyroxine on mitochondrial function. Martius and I found that with small concentrations of thyroxine (10^{-6} M) the phosphorylating activity of rat liver mitochondria was conserved over a period of 16 hr. in the cold in comparison to the control which lost an appreciable amount of activity. This effect could be due to an inhibition of a latent ATP-ase activity, which is activated by ageing or chemical actions like DNP or Mg^{2+} (*Biochem. Z.* **326**, 191 (1955)). On the other hand, I am wondering about the physiological significance of the action of thyroxine or triiodothyronine on the mitochondrial ATP-ase, because we found that triiodothyronine acts on the cytochrome region of oxidative phosphorylation in digitonin particles of rat liver mitochondria. The particles were prepared free from ATP-ase and myokinase and no ATP-ase activity was released by the addition of the hormone. The synthesis of ^{32}P -ATP in the presence of ascorbate, cytochrome *c*, ADP and inorganic phosphate, being activated (max. 35%) by triiodothyronine in the concentration range of 5×10^{-7} M, responds with half maximal inhibition to the presence of 5×10^{-6} M triiodothyronine (unpublished experiments). Perhaps, in the light of the data of Prof. Lindberg and ourselves it seems that there can be a common site of hormonal action upon various components of the respiratory chain, whose elucidation depends upon the experimental conditions. I am wondering whether you have data which shed light on the vertebral level of the hormonal action. Is there a phosphate requirement in your diaphorase experiments?

LINDBERG: No. There is no phosphate requirement.

CHANCE: I was very glad to hear Prof. Lindberg mention that the effect of succinate on DPN reduction might be involved in thyroxine action. Hollunger and I did investigate how much thyroxine would be required to inhibit half maximally the effect of succinate on DPN reduction and found that in the absence of magnesium and without an incubation period of over a minute, that less than 10^{-6} M thyroxine gave half maximal inhibition with a protein concentration of about one mg. per ml., so it is extremely sensitive to thyroxine. I don't know whether this is the primary site of thyroxine action.

LINDBERG: It is our feeling that it is difficult to reproduce experiments with thyroid compounds on respiration and phosphorylation, but the closer you come to a certain region, the diaphorase region, in the respiratory chain the easier it is to get a good, consistent result.

Components of the Energy-Coupling Mechanism and Mitochondrial Structure*

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In this paper approaches taken in our laboratory to the isolation and identification of active mitochondrial proteins involved in the mechanism of respiratory energy-coupling and in the mitochondrial swelling and contraction cycle will be described. This information is still only fragmentary, but it gives increasing hope that the mechanism of oxidative phosphorylation, the structure of the mitochondrial membranes, and the physical nature of swelling and contraction may be studied in molecular terms and that these entities may ultimately be at least partly reconstructed *in vitro* from the isolated catalysts.

Although considerable progress has been made by biochemists in the study of the mitochondrion actually much of this work has involved study of the more organized physiology of the mitochondrion, its control mechanisms, and the action of hormones and drugs. The major reason, without question, for our lack of specific molecular knowledge of mitochondrial chemistry is the fact that the most interesting of the mitochondrial reactions take place in what I once designated as a "solid-state enzyme system" [1], namely the insoluble complex lipoprotein structure of the mitochondrial membranes, which contain the assemblies of respiratory enzymes. Since the first efforts of Warburg and Keilin many years ago, the respiratory enzymes in these membranes have been found to be remarkably refractory to isolation in soluble, homogeneous form. The relatively limited information we now have available on the respiratory carriers and coupling enzymes, as isolated molecular entities, has figuratively had to be carved out of solid rock. The direct approach to chemical study of the molecular components of the mitochondrion has thus been forbidding and frustrating, and has given rise to development of indirect methods and sometimes less than direct study objectives.

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Respiratory chain phosphorylation, the most prominent mitochondrial activity, is not only a very complex enzyme system, but one which differs from many other enzyme systems in that it may never be understood in mechanism until the active sites of the individual enzymes are identified, since there are apparently no low-molecular weight diffusible intermediates. The active sites of the enzymes involved are thus the "intermediates". No amount of indirect experimentation or descriptive, physiological study of the mitochondrion can thus replace direct isolation and chemical study of the catalysts of energy-coupling and the catalysts activating the swelling-contraction cycle.

The three most conspicuous properties of mitochondria are (*a*) the catalysis of respiration and energy coupling, (*b*) the occurrence of reversible swelling and contraction, leading to water movements, which are geared to respiration and (*c*) ion transport, also geared to the respiratory chain. After considering the mitochondrial membranes, in which these functions apparently reside, this paper will deal with recent work on some isolated components involved in these functions.

Molecular organization of the mitochondrial membranes

It is now clear from many items of evidence that the enzymes of respiration and coupled phosphorylation are more or less firmly embedded in or on the mitochondrial membranes; indeed circumstantial evidence suggests that the inner membrane which presumably forms the cristae is the site of these enzymic activities. When the mitochondria are subjected to disruption by either digitonin or sonic oscillation, they shatter into fragments having a wide spectrum of particle weights. We have examined the enzymic properties of a series of such fragments differing in sedimentation rate and have found that they have a fairly constant content per mg. protein N of cytochrome oxidase, β -hydroxybutyric dehydrogenase, succinoxidase, and ATP-ase, regardless of particle size [2], suggesting that the membranes are made up of a large number of recurring structural units, each of which may contain a complete assembly of respiratory carriers in finite ratio, as determined by difference spectra.

Calculations suggest that an individual liver mitochondrion may contain 5000–10 000 or more, of such respiratory assemblies, which are more or less evenly distributed on the membrane. Extension of such calculations, with certain assumptions, indicates that a large fraction of the total mass of the membrane is made up of these assemblies of catalytically active molecules—perhaps as much as 40% by weight [2]. The membranes also contain considerable "phosphoprotein" and the phosphate groups undergo replacement at a high rate. The exact disposition of protein and lipid molecules in the membranes is not yet clear. The original

concept of the structure proposed by Sjöstrand is now under some modification and refinement by other workers, notably Robertson [3]. In any case, alternation of oriented lipid and protein molecules in unimolecular layers appears to be the basic structural plan.

In the light of these considerations it is clear that the permeability and physical state of the mitochondrial membranes could logically be expected to be functions of the activity or state of the catalytically active proteins which apparently make up such a large part of the structural mass of the membranes. Thus the swelling-contraction cycle of the membranes and their characteristic selective permeability may be attributed to mechano-chemical changes of the respiratory and coupling enzymes, analogous to the mechano-chemical activities of the actomyosin complex. Furthermore work of Gamble in our laboratory [4] has demonstrated that the membranes are also the site of perhaps the most prominent reaction of mitochondrial active transport, namely the active binding of K^+ . Isolated digitonin fragments of the membranes bind K^+ specifically during coupled phosphorylation to an extent which can account nearly completely for the entire activity of intact mitochondria.

Finally it should be pointed out that the selective permeability of the mitochondrial membranes may be an element in physiological control mechanisms. For example, it has been assumed in some recent speculations on the mechanism of the Pasteur reaction [5] that ATP generated by mitochondria is segregated or compartmented in the mitochondria, so that it does not "mix" with glycolytically generated ATP.

"Partial reactions" and the mechanism of oxidative phosphorylation

No attempt will be made to review in any detail the development of ideas and the experimentation which have led to current outlines of knowledge; recent reviews by Slater [6], Lehninger [1, 2, 7, 9, 10], and Chance [8] may be referred to. However, some of the most valuable information has come from study of the so-called "partial reactions" of oxidative phosphorylation which are reflections of the fact that at least some if not all the intermediate reactions are reversible. The most fundamental discovery was probably the finding that the uncoupling agent dinitrophenol stimulates hydrolysis of ATP, indicating that a "leak" in the coupling mechanism occurs in the presence of this reagent (cf. [11]). Since DNP can release respiration from its dependence on ADP in the absence of inorganic phosphate (cf. [6]), the site of action of DNP appears to be at a point prior to the uptake of phosphate.

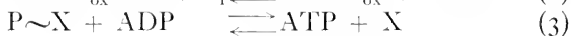
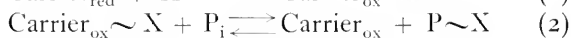
A second "partial reaction" of great significance is the $ATP \rightarrow P_i$ ³² exchange. In the absence of net electron transport the terminal phosphate

group of ATP exchanges very rapidly, a reaction which is completely inhibited by dinitrophenol [12].

Both the ATP-ase and ATP-P_i³² exchange have been studied to greatest advantage in so-called digitonin fragments of the membranes of rat liver mitochondria [2, 13], which contain complete respiratory chains and coupling mechanisms but do not show Krebs cycle activity. These fragments are relatively free of enzymes not relevant to oxidative phosphorylation and are not so subject to compartmentation phenomena as are intact mitochondria. With these fragments it was found that the partial reactions are specific for nucleotides of adenine. Further, the requirements and kinetics of the ATP exchange reaction could be examined more closely. It was found that ADP was a necessary component in the ATP-P_i³² exchange [14] and also that this exchange was most rapid when the respiratory carriers were in the fully oxidized state [15].

During further examination of the mechanism of the ATP-P_i³² exchange, it was found that digitonin preparations also catalyze an exchange of labelled ADP into ATP which was inhibited by DNP [14, 16]. This exchange, which is specific for adenine nucleotides, does not require inorganic phosphate and was found not to be caused by other phosphate-transferring enzymes known to catalyze ATP-ADP exchanges, such as adenylate kinase and protein phosphokinase. The exchange activity is stable but on ageing loses its sensitivity to DNP. This striking finding was corroborated by independent experiments with azide; this agent does not affect the rate of the ATP-ADP exchange but prevents it from being inhibited by DNP. The tentative conclusion was drawn that the ATP-ADP exchange reaction is a reflection of the action of the terminal enzyme of oxidative phosphorylation, but that this enzyme is not itself sensitive to DNP. However, it was postulated that its sensitivity to DNP was conferred on it because it is in equilibrium with a preceding reaction in the coupling sequence which has a DNP-sensitive component.

The information on the ATP-ase activity and the phosphate and ADP exchange reactions therefore suggested that the general form of the energy-coupling reactions could be expressed by the following equations [2, 14, 15]:



Reaction (3) thus accounts for the ATP-ADP exchange, reactions (2) + (3) for the ATP-P_i³² exchange, and the sequence of reactions 3 + 2 plus the following reaction (4) for DNP-stimulated ATP-ase activity:



While this represents the simplest statement, as will be seen it is possible that one or more additional intermediate reactions may also occur. The above sequence accounts for the finding that ADP is necessary for incorporation of P_i into ATP and that P_i is not necessary for incorporation of ADP into ATP [2]. It is suggested that this basic mechanism occurs at all three phosphorylation sites of the respiratory chain, but it is not yet known to what extent each of the three sites contributes to the overall rates of the partial reactions. The outline of the reaction pattern described here is in general consistent with most experimental observations, but there have been some differences in interpretation which are fully outlined by Slater [6]. The value of any hypothesis is the fruitfulness of experimentation which it may suggest.

Separation of the ATP-ADP exchange enzyme

It was found that the relatively stable enzyme catalyzing the ATP-ADP exchange could be extracted from acetone powders of digitonin fragments and of mitochondria in soluble, highly active form and in nearly complete yield [16]. It has now been purified approximately 150-fold by Dr. Charles L. Wadkins, using ammonium sulphate fractionation and chromatography on cellulose columns. While a minor component is still present, preliminary examination indicates that the protein is of relatively small molecular weight and that it is free of lipid. The highly purified enzyme requires Mg^{++} or Mn^{++} for activity, is quite stable to dialysis and storage, and is reversibly inhibited by *p*-chloromercuribenzenesulphonate (PCMB). It has been assayed for activity in promoting other phosphate-transferring reactions which are known to bring about ATP-ADP exchanges, such as myokinase and protein phosphokinase, but such activities are absent. In addition the enzyme does not show ATP- P_i ³² exchange activity or ATP-ase activity, in the presence or absence of DNP. The enzyme is not identical with that described by Chiga and Plaut [17] who have obtained a highly purified enzyme from heart mitochondria catalyzing both the ATP- P_i ³² exchange and ATP-ADP exchange and which is most active with Mn^{++} .

Recombination of soluble ATP-ADP exchange enzyme with digitonin fragments

The soluble form of the ATP-ADP exchange enzyme is completely insensitive to dinitrophenol. In this form it therefore possesses no distinctive characteristics which identify it as a portion of the energy-coupling machinery of oxidative phosphorylation. Experimental approaches were therefore taken to establish more firmly the relevance of this enzyme in

its soluble form to the mechanism of respiratory energy coupling. In intact mitochondria [18] or membrane fragments [16] the ATP-ADP exchange is sensitive to DNP, but only indirectly, as pointed out above. If the soluble ATP-ADP exchange enzyme could be "reconnected" with the DNP-sensitive reaction, the soluble enzyme might regain its DNP-sensitivity. We have now found it possible to reconfere DNP-sensitivity on the soluble form of the ATP-ADP exchange enzyme quite simply by adding it to fresh preparations of digitonin fragments [19]. The typical experiment illustrated in Fig. 1 shows that addition of the DNP-insensitive, soluble exchange enzyme to fresh rat liver digitonin fragments in which the

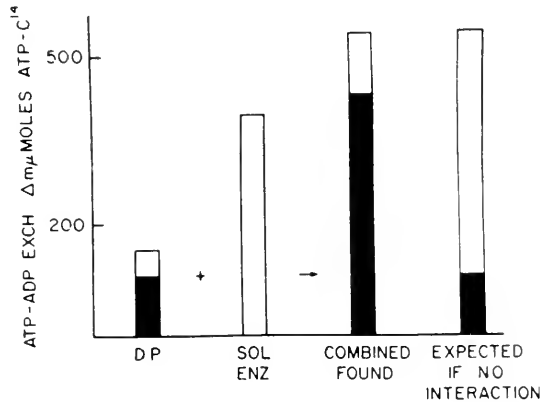


FIG. 1. "Recombination" of soluble, DNP-insensitive ATP-ADP exchange enzyme with digitonin particles to restore DNP-sensitivity. System contained 0.01 M ATP, 0.006 M [14 C]-ADP, and 5×10^{-4} M DNP where shown. Black portion of bars indicates fraction of activity sensitive to DNP.

inherent nucleotide exchange activity is inhibited significantly by dinitrophenol, causes, in the combined system, a summation of the total exchange activities in the absence of DNP. However, it is evident that a very large fraction of the combined ATP-ADP exchange activity is now sensitive to dinitrophenol, to a far greater extent than would be expected by simple addition of the two reactions measured separately. Many experiments of this kind thus demonstrate the conferral of DNP sensitivity on the soluble nucleotide exchange reaction by adding it to fresh digitonin particles. Dinitrophenol sensitivity is not conferred on the soluble enzyme by aged digitonin particles, which are incapable of oxidative phosphorylation (Fig. 2). Furthermore the DNP-sensitivity of the recombined system is abolished in the presence of azide, which, as mentioned above, can dissociate the particulate ATP-ADP exchange from the DNP-sensitive site (Fig. 3). The conferral of DNP-sensitivity on the soluble ATP-ADP exchange enzyme is thus specific and this finding establishes the identity

of the latter with the exchange activity observed in intact mitochondria. Addition of adenylate kinase, which also catalyzes an ATP-ADP exchange, to fresh digitonin particles does not confer DNP-sensitivity on this reaction, for example.

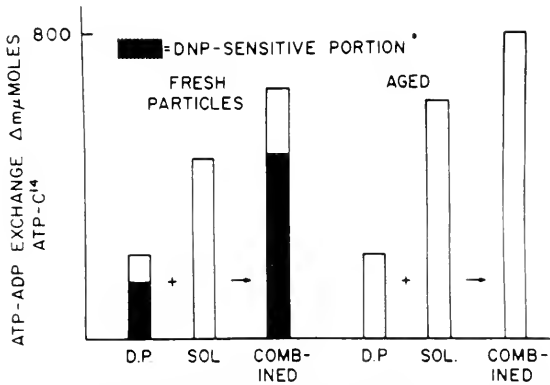


FIG. 2. Failure of aged digitonin particles (48 hr. at 2°) to confer DNP-sensitivity on soluble ATP-ADP exchange enzyme.

We have concluded that the digitonin particles have lost, during the course of preparation, a significant fraction of the molecules of the ATP-ADP exchange enzyme present in mitochondria. However, the binding sites to which these molecules are normally attached are still functional

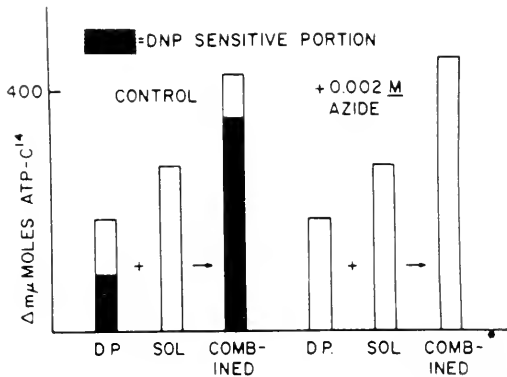


FIG. 3. Effect of 0.002 M azide on "recombination" of ATP-ADP exchange enzyme with digitonin particles.

and capable of "rebinding" the soluble form of the exchange enzyme in a specific manner so as to bring the nucleotide exchange reaction it catalyzes into equilibrium with a DNP-sensitive reaction. In consonance with this conclusion we have found that there is an upper limit to the capacity of

any sample of digitonin particles to "rebind" soluble ATP-ADP exchange enzyme; this upper limit in molar terms is approximately equal to the total potential ability of the preparations to catalyze phosphate uptake at a P:O ratio of 3. The specific rebinding to phosphorylating assemblies indicates that the soluble ATP-ADP exchange enzyme is a part of the coupling machinery. It also indicates that this exchange enzyme has another functional site which is reactive with an as yet unknown "substrate" molecule, presumably a preceding enzyme of the coupling sequence, with which ATP and ADP must come into equilibrium.

In preliminary experiments it has been found that the soluble ATP-ADP exchange enzyme, when added to digitonin fragments giving suboptimal phosphorylation, will significantly increase the P:O ratio [9,20]. While the effect requires further investigation, it gives further evidence for participation of this enzyme in the mechanism of oxidative phosphorylation.

M-factor

With the availability of highly purified preparations of the ATP-ADP exchange enzyme, efforts were begun to establish the nature of the binding of this enzyme to preceding components of the coupling mechanism, in the hope that the chemical nature of the intermediate reactions catalyzed in the energy-coupling sequence could thus be approached. An important lead into the enzymic aspects of the recombination phenomena was afforded by the finding that extracts of mitochondria contain a soluble heat-labile substance of protein nature (designated as M-factor) which when added to normal digitonin particles greatly increases the sensitivity of the inherent ATP-ADP exchange reaction to dinitrophenol [21].

Most preparations of digitonin particles are substantially but not completely inhibited by dinitrophenol [16]. The degree of inhibition, which varies from 20-90% among different preparations cannot be increased simply by increasing dinitrophenol concentrations above the level of approximately 5×10^{-5} M (which produces essentially complete uncoupling of oxidative phosphorylation). This finding suggests that the total ATP-ADP exchange activity of any given sample of digitonin particle consists of two components: a "coupled" component, sensitive to DNP, and a "dissociated" or "uncoupled" ATP-ADP exchange activity, which may be a portion of the coupling machinery but which has been "dislocated" from the DNP-sensitive reaction during preparation of the particles.

Data in Fig. 4 show that addition of soluble partly purified protein fractions from mitochondrial extracts can greatly increase the fraction of the total ATP-ADP exchange activity which is sensitive to dinitrophenol.

This activity, called M-factor, can be assayed semi-quantitatively with the system shown and it has been purified over fortyfold. The starting material is either a phosphate extract of acetone-powdered mitochondria, from which M-factor can be precipitated by relatively low concentrations of ammonium sulphate or, curiously, simple extracts of whole fresh rat liver mitochondria made with 0.3 M ammonium sulphate. M-factor activity from such extracts can then be recovered by further treatment with ammonium sulphate. The M-factor preparations contain essentially no ATP-ase activity, ATP-P_i³² exchange activity or ATP-ADP exchange activity. They are also free of adenylate kinase and protein phosphokinase.

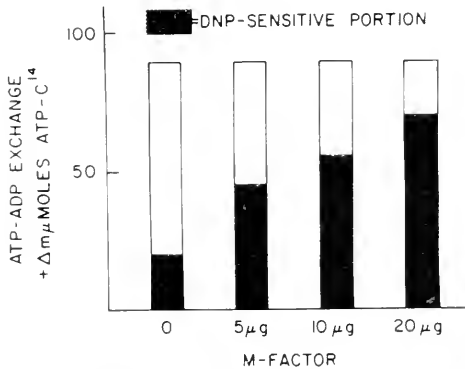
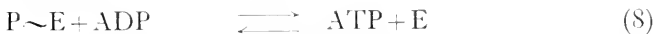
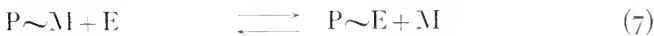
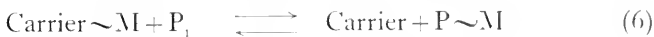


FIG. 4. Increase of DNP-sensitivity of ATP-ADP exchange in digitonin particles by M-factor.

Two possibilities are open for the mechanism of action of M-factor. The first is that M-factor is a specific "cementing" protein capable of binding with the ATP-ADP exchange enzyme molecule in such a manner as to hold it in the appropriate geometry on the digitonin particle so that it may become reactive with the preceding, DPN-sensitive reaction. On the other hand, M-factor may itself be an intermediate enzyme of the energy-coupling mechanism. A possible mode of action is given in the following equations:



in which E represents the ATP-ADP exchange enzyme and Carrier ~ M the high-energy complex of the carrier generated during electron transfer. M thus could be visualized as replacing the X of the earlier formulation

given above. M-factor therefore could serve as an intermediate enzyme in the respiratory energy coupling sequence, which is capable of transferring high energy groups from the coupled carrier to the terminal enzyme E catalyzing the ATP-ADP exchange reaction. This possibility is being examined directly (a) by studying the participation of M-factor in the binding of external soluble ATP-ADP exchange enzyme to the presumably empty sites in digitonin particles, to determine the sequence and stoichiometry of rebinding, and (b) by examination of complex formation between M-factor protein and the ATP-ADP exchange enzyme by physical methods and by kinetic approaches. Recently we have found that when the soluble ATP-ADP exchange enzyme is more highly purified, it no longer can "recombine" with digitonin fragments to restore DNP-sensitivity. It appears possible that purification has removed a factor necessary for "recombination" and work is in progress to determine whether M-factor is involved in binding soluble ATP-ADP exchange enzyme.

Relationship to other soluble factors supporting oxidative phosphorylation

While many investigators have observed that soluble protein fractions, particularly from bacterial extracts, can increase the P:O ratio of respiratory chain preparations (cf. [22, 23]), in general, little is known of the enzymic capabilities of such soluble fractions and the contribution they make to the overall coupling mechanism. Similarly the protein fraction isolated by Titchener and Linnane from beef heart mitochondria [24], which increases the P:O ratio of pretreated beef heart particles, is of relatively unknown enzymic competence. However, the important work of Pullman, Penefsky, and Racker [25] has shown that a highly purified soluble enzyme catalyzing DNP-stimulated ATP-ase in the presence of Mg^{++} , increases the P:O ratio of mechanically disrupted beef heart mitochondria. This factor, which shows extraordinary lability to cold, does not catalyze the ATP- P_i^{32} exchange reaction or an ATP-ADP exchange reaction. While this soluble ATP-ase seems not to be identical with either our ATP-ADP exchange enzyme or the M-factor described above, at least on superficial comparison of properties, yet it cannot be excluded that there are elements of identity. It is possible for example that a complex of the ATP-ADP exchange enzyme and M-factor may be equivalent to the Pullman ATP-ase, at least in some respects. In any case further development of both lines of work and comparison of the findings should be of great importance. It must be recalled that there are three phosphorylation sites in the respiratory chain. While it is comforting to think that all three operate by the same mechanism, this need not be the

case and it is therefore possible that the two laboratories are studying reconstruction of different phosphorylation sites in the chain.

Of greatest importance, however, is the fact that it now seems possible to obtain from mitochondria in soluble and fairly stable condition, specific protein factors which appear to be concerned in the mechanism of respiratory energy coupling. Identification of the specific enzymatic capabilities of these reactions may represent a "breakthrough" to real understanding of the mechanism of oxidative phosphorylation. It is of course quite possible that current hypotheses on the mechanism of phosphorylation and the postulated role of these factors are wrong. However, the important thing is that these soluble factors are now at hand and that they can be examined more carefully at the molecular level in reconstituting oxidative phosphorylation.

The swelling-contraction cycle of mitochondria

An independent approach to the mechanism of oxidative phosphorylation comes from work on the contraction of mitochondria. Abundant evidence now exists that both the swelling of mitochondria and their active contraction, leading to uptake and extrusion of water respectively, are phenomena which are geared to the activity or state of the respiratory carriers and/or the energy coupling mechanism by which ATP is formed in mitochondria (cf. [26]). The enzymes of respiration and phosphorylation are located in the membranes and are thus in a strategic position to provide mechano-chemical control over membrane properties, such as their molecular geometry and their permeability. In the following discussion, swelling and contraction will refer to those changes in membrane properties specifically associated with the respiratory chain and the coupling mechanisms which can lead to changes in the mitochondrial volume. The transitory and purely osmotic changes which can be effected in mitochondrial volume for some seconds on altering merely the osmotic pressure of the medium with solutes of varying degrees of penetrability [27] will not be discussed here. Such properties are of course common to all structures bounded by semipermeable membranes.

That mitochondrial swelling is a function of the activity of the respiratory chain is shown most strikingly by the finding that swelling is inhibited by respiratory inhibitors such as amytal, antimycin A and cyanide or by simple anaerobiosis [28-30]. These factors inhibit swelling induced by a variety of agents (cf. [26, 31]) such as phosphate, thyroxine, calcium, phlorizin, and many others. At first it was concluded that this inhibition was due to the maintenance of the carriers in the reduced state, particularly DPNH [28, 29]. However, more extensive work by Chappell and Greville [32] has shown that it is more likely that mitochondrial swelling requires

occurrence of active electron transport which may be afforded by endogenous substrates. The susceptibility to swelling agents can be conferred by electron transfer in different segments of the respiratory chain. On the other hand, data of Birt and Bartley among others, suggest [33] that both the oxidation-reduction state of the carriers and the net electron flux may be elements in susceptibility of mitochondria to swelling.

Another piece of evidence implicating the coupled respiratory chain in the swelling process is that sucrose and other polyhydroxylic compounds inhibit swelling [29, 34]. These compounds also inhibit respiration and uncouple phosphorylation in the osmotically insensitive digitonin fragments of the mitochondrial membrane, suggesting they act as enzyme inhibitors rather than in an osmotic sense [35]. In addition, dinitrophenol has been found to inhibit swelling when added to fresh mitochondria [36], whereas on delayed addition it becomes an activator of swelling [37].

Mitochondrial swelling *in vitro* induced by thyroxine or phosphate leads to an increase of volume of between 100 to 200 per cent over a period of 10–15 min. at 20°. Small-amplitude swelling of tightly-coupled mitochondria has also been found to be dependent on respiration or respiratory state by Holton [38] and Packer [39].

In large amplitude mitochondrial swelling taking place over longer periods, a large part of the respiratory control by ADP is lost, as well as ability to phosphorylate, possibly as a consequence of the "stretching" of respiratory assemblies in the membranes. However, as is shown below, such drastic mitochondrial swelling is still reversible by ATP [34, 40].

Mitochondrial contraction

Price *et al.* first established in their thorough study [41] that re-institution of phosphorylating respiration in swollen mitochondria by appropriate supplements to the test medium would cause a contraction with gravimetrically measurable extrusion of water. Similar observations were reported by Beyer *et al.* [42]. Since the mechanism of oxidative phosphorylation, at least in its terminal stages, has been thought to be reversible, it would have appeared likely that ATP alone in the absence of respiration might be able to effect mitochondrial contraction. However, with the exception of a very limited contraction observed by Chappell and Perry in pigeon breast muscle mitochondria by the addition of ATP [43], no significant success was reported in effecting contraction by mere addition of ATP to swollen mitochondria. In 1959 we found that the failure of ATP to effect contraction could be traced to the presence of sucrose in the test media ordinarily used in such experiments [29, 44]. Sucrose in approximately isotonic concentrations completely inhibits contraction of swollen liver mitochondria by ATP, whereas mitochondria

contract quite well in a buffered KCl medium on addition of ATP. The addition of ATP + Mg^{++} + serum albumin was found to cause immediate contraction of mitochondria swollen by a wide variety of agents, including thyroxine, oleate, phlorizin, calcium, PCMB, phosphate, and many others [44] and, curiously, mitochondria swollen by digitonin and by carbon tetrachloride. Actual extrusion of water was demonstrated by gravimetric methods to accompany the optical changes. It was shown that several hundred moles of water could be extruded per mole of ATP split [34].

The molecular mechanism of the ATP-induced contraction can be shown to be completely independent of respiration and net phosphorylation, since it proceeds perfectly well in a medium containing sufficient

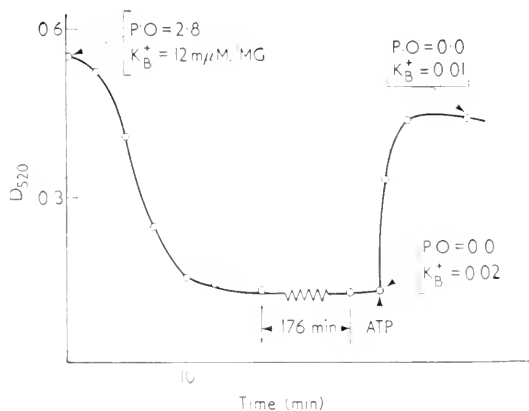


FIG. 5. Independence of ATP-induced contraction from oxidative phosphorylation and K^+ binding. Allowing mitochondria to stand in swollen state at 25° for extended periods abolishes phosphorylation and K^+ -binding, without affecting ability to contract.

cyanide or other respiratory inhibitors to block respiration or in the presence of sufficient dinitrophenol to completely uncouple oxidative phosphorylation, as long as ATP is in excess [34]. On the other hand, it is clear that the ATP-induced contraction must employ at least a portion of the energy coupling machinery, since this contraction is blocked by inhibitors such as azide, which disconnects the ATP-ADP exchange reaction from the dinitrophenol sensitive site, and is also inhibited characteristically by sucrose and many other sugars and polyhydroxylic alcohols, which are also known to inhibit oxidative phosphorylation and the ATP- P^{32} exchange reaction as well as ATP-ase [35]. Furthermore, contraction of mitochondria by ATP is not dependent on any specific ionic environment and can occur in mitochondria whose ability for K^+ transport is completely inactivated [40] (Fig. 5).

It appears likely that mitochondrial contraction induced by ATP

causes extrusion of small solute molecules along with water. On the other hand, since swollen mitochondria are still relatively impermeable to large molecular weight compounds such as serum albumin and polyvinylpyrrolidone [29], the soluble proteins and other high molecular weight substances in the intramitochondrial space probably do not leave during contraction. If this is the case, then osmotic work is carried out during ATP-induced contraction, because it leads to a more concentrated solution of the high molecular weight solutes inside the mitochondria.

Preliminary examination of thyroxine-swollen and ATP-contracted mitochondria with the electron microscope [45] shows the swollen mitochondria to be very large and spherical, containing large optically clear vesicles and few or no recognizable cristae. After contraction, they are much smaller, optically dense, contain no vesicles, and show nearly normal cristae.

Swelling and contraction of mitochondria therefore clearly involve the respiratory chain and the associated energy coupling mechanisms, but the two phases employ or are activated by different segments or portions of this complex enzymic machinery. Swelling requires the action of the respiratory chain, but the contraction does not; however, terminal stages of energy coupling appear to be involved in the latter phase. The swelling and contraction therefore appear not to be reversible in the sense that they employ reversibly the same controlling catalysts. Furthermore, because of the occurrence of two mitochondrial membranes it is possible that swelling may be a function of the properties of the inner membrane, for example, and contraction a function of the outer membrane, since all kinds of mitochondrial swelling can be contracted again by ATP [40].

Because sucrose and other polyhydroxylic compounds such as glucose, raffinose, fructose, dextran, xylose, mannitol, and sorbitol in concentrations of 0.1 M to 0.6 M inhibit both swelling and contraction (the latter more strongly), as well as ATP-ase and the ATP-P_i³² exchange in osmotically insensitive digitonin particles, we have suggested that these compounds are efficacious in preserving mitochondrial morphology during isolation more for their ability to act as inhibitors of an intermediate enzymic reaction involved in the swelling-contraction cycle than for their relative slowness of penetration [29, 35]. Simple alcohols or compounds like ethylene glycol and glycerol do not inhibit. It has been suggested that the polyhydroxylic alcohols act as artificial acceptors in "transferase" reactions, displacing the normal group acceptor.

Biochemistry of the contractile process

It now seems possible to approach chemical analysis of the mechanism of contraction. A guiding principle for such approaches is the hypothesis

that the swelling and contraction are reflections of the action of "mechano-enzyme" systems similar to the actomyosin of muscle, in which intermediate enzymes of the energy-coupling mechanism may act as "mechano-enzymes" and undergo change of shape or charge distribution. If the ATP-ADP exchange enzyme can exist in phosphorylated form, this might differ in configuration or in geometrical arrangement from the unphosphorylated form and account for changes in the geometry or properties of the membrane [26, 34]. There is in fact a striking resemblance between the ATP-ase activity of the actomyosin system and that of the phosphorylation mechanism.

Mechanisms of membrane changes

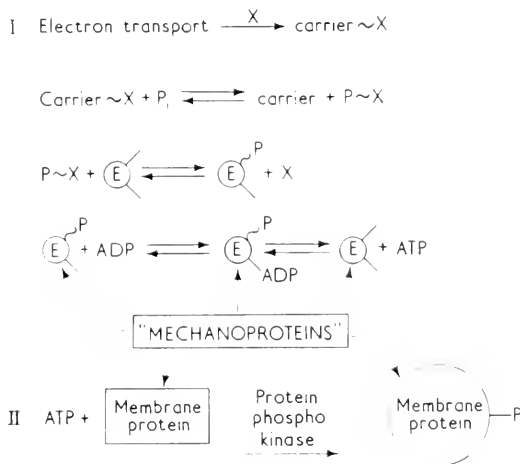


FIG. 6. Two possible mechanisms for alteration of membrane state through ATP-driven changes of shape or conformation of protein molecules. In the first, the mechano-enzyme may be an intermediate enzyme of energy coupling, such as E whose shape may change as a function of binding of P or ADP or ATP. In the second, an independent membrane protein (possibly the "phosphoprotein" of mitochondria) may be activated by ATP to yield mechanical changes.

Figure 6 indicates two possible ways in which contraction might be visualized. In the upper half is shown a representation in which an intermediate enzyme of the energy-coupling sequence is the "mechano-enzyme" activating the contractile changes. It is postulated to change shape or charge distribution when it is phosphorylated.

On the other hand, it is possible that the contractile protein is not a member of the coupling sequence itself but perhaps is in equilibrium with it. We have suggested that the "phosphoprotein" of mitochondria is a possible candidate, since earlier work with Friedkin had shown that the

phosphorus of this fraction has a significantly high rate of turnover [46]. Although preliminary work by Dr. Ishikawa as a test of this possibility appeared very promising, because the mitochondria contain a protein phosphokinase [47], analytical difficulties of an unexpected nature still prevent a clear-cut evaluation.

However, a rather different development provided an important approach to the chemistry of contraction. This was the finding that mitochondrial swelling induced by reduced glutathione is different from swelling caused by other agents such as phosphate or thyroxine in its kinetics and in its control [48]. Furthermore, swelling induced by glutathione is not reversed by ATP under the same conditions which can reverse swelling caused by other agents. It was soon found that this failure of contraction was due to the detachment from the mitochondria of a necessary

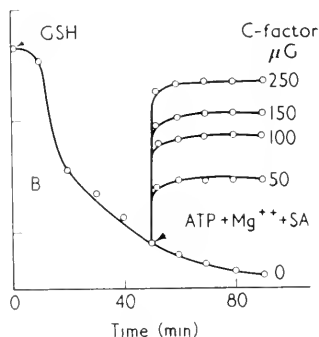


FIG. 7. Requirement of C-factor for contraction of glutathione-swollen mitochondria.

protein factor (designated C-factor) on exposure to glutathione [49]. This factor leaked out into the medium and could be recovered. Only when this factor was added back in appropriate concentrations to the test medium could contraction of the mitochondria be observed in the presence of ATP + Mg⁺⁺ + BSA. The C-factor can be assayed, as is shown in Fig. 7, by the level of contraction achieved as a function of the concentration of the factor in the medium. With this simple bioassay it was found possible to demonstrate the occurrence of C-factor in sonic extracts of mitochondria and in extracts of digitonin particles. It was found not to be dialyzable, it is labile to heat, and survives acetone drying or lyophilization. It has now been purified over fifty-fold by Dr. Diether Neubert.

Recently we have carried out an examination of C-factor activity in different tissues and in different tissue fractions [50] with the surprising finding that this factor is found not only in mitochondria but also in *extra*-mitochondrial cytoplasm. C-factor activity has been found in the mitochondria and extra-mitochondrial cytoplasm of a number of tissues of the

rat, in Ehrlich ascites tumour cells, and also in erythrocytes and extracts of *Escherichia coli*. Of great significance is the finding that C-factor activity is especially rich in contractile tissues like skeletal muscle and cardiac muscle. We have earlier called attention to the possibility that C-factor may bear a relation to the mitochondrial membrane analogous to that born by actin to myosin. While this analogy is only suggestive at this stage, it is of interest to point out that erythrocyte membranes can be induced to change shape in the presence of ATP [51] and it is now well known from studies of Abrams and others that bacterial protoplast membranes also undergo swelling-contraction cycles which are metabolism-dependent [52].

It is also significant that the partly purified specimens of C-factor contain some ATP-ase activity, which suggests that they may be related to the factor described by Pullman *et al.* [25], which is capable of restoring oxidative phosphorylation in heart preparations.

Other factors in mitochondrial contraction

There is evidence that mitochondrial substances other than C-factor are necessary in contraction. It has been found by measuring the light-scattering envelope of intact mitochondria [53] that the ratio of light scattered at 135° to that scattered at 45° to the incident beam measures a change in mitochondrial configuration induced by ATP which is not measureable by simple light absorption or by light scattered at 90° . This change is promoted by substance(s) "leaking" from mitochondria stored simply at 0° in sucrose which are apparently not identical with C-factor.

Lastly, the rather puzzling effect of L-thyroxine in stimulating mitochondrial contraction by ATP [54] must be mentioned. L-thyroxine is thus not only a swelling agent, but can also stimulate contraction.

Concluding remarks

A number of soluble mitochondrial factors having significant action of an apparently enzymic nature on oxidative phosphorylation and mitochondrial swelling and contraction have now been recognized. These include (1) the soluble ATP-ADP exchange enzyme, (2) M-factor, (3) C-factor, (4) sucrose-extracted contraction factor, as well as earlier described entities such as (5) U-factor [55] (presumably an uncoupling fatty acid) and (6) R-factor, a protein fraction which releases respiration from its dependence on ADP but which does not uncouple phosphorylation [56]. With the protein factors from beef heart mitochondria separated by Titchener and Linnane [24] and by Pullman *et al.* [25], as well as the increasing successes in dissociation and recognition of the respiratory carriers, a significantly large number of elements of the mitochondrial membrane

are recognizable and are susceptible to assay, purification, and use in reconstruction experiments. Furthermore, applications of physical methods to isolated proteins of the membrane, particularly the coupling enzymes, may provide direct approaches to study of the "mechano-enzyme" nature which we have postulated to account for the swelling-contraction phenomena.

While our knowledge of oxidative phosphorylation and of the mechanism of mitochondrial swelling and contraction is still fragmentary and there are many loose ends still to be accounted for satisfactorily, it is evident that these complex chemical and mechano-chemical activities of the mitochondrial membrane are approachable on the molecular level and can be at least partly reconstructed or reconstituted. It is of course important to examine these phenomena as they occur in intact mitochondria because of the extraordinary possibilities they afford for physiological control mechanisms, however it is clear that the greatest challenge and the most significant developments toward full knowledge of the molecular biology of the mitochondrion can be expected to come from examination of the separate molecular entities participating in these complex reactions.

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Discussion

ESTABROOK: Dr. Fugmann and I have studied the reactions of the endogenous pyridine nucleotides of the digitonin particles and we find that the extent of reduction of the pyridine nucleotide during the steady state is largely dependent on the presence of versene. In other words one cannot get a cyclic response of the pyridine nucleotide with ADP without versene being there. Have you tried any of these metal chelators or do you have any indication that the action of the M-factor has any relation to chelating properties? My second question is: according to your second mechanism, phosphate itself should serve as an uncoupler because it will liberate high concentrations of free carrier. I wonder if you have any explanation for this point.

LEHNINGER: With regard to your first question I should have mentioned that for the action of the M-factor we need Mg^{2+} so possibly some function of the metal is required here, but the effect is not given by EDTA. With regard to your second question, I don't think that the mechanism I had on the board is the last word on the mechanism of phosphorylation but you have to start somewhere with a working hypothesis. The value of any of these hypotheses is, I think, just a matter of how many crucial experiments you can design based on them. In the case of the second mechanism, phosphate could act as an uncoupling agent only if $P \sim M$ is split by either hydrolysis or reaction with E, to regenerate free M, which is required for respiration as well as the carrier. Since the molar amount of E is limited, free M could be regenerated only if free E could be regenerated. Arsenate can uncouple because the intermediate arsenylated compounds are presumably unstable.

CHANCE: I was very interested in Dr. Lehninger's discussion of the mechano-protein which I think is a very important concept, especially in view of the work of Pullman and Racker. However, being part physical chemist, there is a critical question which I can ask, that is whether the time-course of contraction of the hypothetical mechano-protein in mitochondria is one that is compatible with its function in oxidative phosphorylation. It is true that the ADP-induced light scattering increase on contraction, observed by Packer and myself, is a rapid one and is possibly compatible with the mechano-protein idea, but it seems to me that it is the opposite sense to the ATP-induced contraction, which also seems to me to be on a slower time scale. Are there two kinds of mechano-proteins, one studied in the presence of ATP and on a fairly long time scale, and one observed on ADP?

LEHNINGER: In our earlier publications it appeared that ATP-induced contraction was quite slow; this was because we did not understand the optimum conditions. Now on more refined investigation I am not sure that there is a great difference in the speed of ATP- and ADP-induced contraction. I don't think there is any great disparity there, but there is no doubt that the changes that we and Packer observed lag behind the changes in the steady state of the carriers. However, I don't think this is completely incompatible with the picture I have drawn for the following reason: the morphology of the mitochondrion is pretty complicated, and it is possible that in ATP- or ADP-induced contraction there is a sequence of events. First, in the primary event a given molecule changes shape or contracts in the presence of ATP, possibly synchronous with change in oxidation-reduction

state. However after the primary event there are certainly secondary events, which follow the ATP-induced change, but it may take a certain finite lag period before the rest of the very complicated mitochondrial structure undergoes those changes which register as total light scatter. Does that make sense?

CHANCE: The facts are that we don't have fast ADP changes and the ATP ones are slower.

JAGENDORF: When you restore DNP sensitivity to ATP:ADP exchanges by mixing the isolated enzyme with fresh particles do you have to deplete these particles first?

LEHNINGER: No. They are already partly depleted. We can deplete them further by exposing them to high salt concentrations or to glutathione.

JAGENDORF: Could this M-factor be a polynucleotide as in Pinchot's experiments?

LEHNINGER: Although M-factor is a heat-labile, non-dialysable substance, it is possible that it carries a bound polynucleotide. We have tried polynucleotides and at least with the polyadenylic acid and polyuridylic acid specimens we had they did not work.

MITCHELL: I was very interested in Dr. Lehninger's concept of a mechano-protein, but I would suggest that we need to take more care not to be too romantic about this concept. We heard in the first discussion of this symposium (Dr. Kendrew) how, during the uptake of oxygen, the haemoglobin molecule can become reorientated and change shape; and I suggest that in thinking about mechano-proteins we should bear this example in mind as it illustrates the principle of change of orientation implicit in the mechano-protein conception and is free of the potentially misleading associations of the usual elastic catapult kind of model. Perhaps it would be better to use the phrase "mechano-protein complex", because we do not want to give the feeling that a change of shape and the contraction which results is necessarily due to the contraction of individual protein molecules as we used to think in the case of muscle.

LEHNINGER: I completely agree that we should not be too precise about specifying the mechanism. You are quite right, I would regard haemoglobin as an example of a mechano-protein in this very general way. Obviously changes in charge distribution or a dissociation are also possible molecular mechanisms under this generic name.

AZZONE: You have tried to calculate the stoichiometry between the moles of H_2O extruded from the mitochondria and the moles of ATP split. I wonder whether it is necessary for ATP to be hydrolyzed or merely to be bound to the mitochondrial membrane. Do you think it is possible to inhibit the ATP-ase activity and still maintain the ATP-induced contraction of the mitochondria?

LEHNINGER: As I said we are not prepared to state that ATP-ase activity is necessary for the contraction; contraction may require only binding of ATP, just as in Morales' theory of contraction of actomyosin. Secondly, we have not found any inhibitor or circumstances where we can inhibit ATP-ase without also inhibiting contraction.

Ascorbate-Induced Lysis of Isolated Mitochondria— A Phenomenon Different from Swelling Induced by Phosphate and Other Agents*

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Many substances induce swelling in isolated liver mitochondria. Relatively few substances other than surface active agents such as the detergents produce a lytic type of effect. In the course of earlier work [1] we observed that low concentrations of ascorbate have a characteristic lytic effect. This phenomenon has been studied to establish the nature of the reaction, to obtain clues on the key groups in the mitochondrial membrane, and to explore the possibility of preparing submitochondrial particles or units and soluble proteins in this way.

Glutathione and cysteine produce effects which appear to be similar to but not identical with those seen with ascorbate. Feldott, Johnson, and Lardy [2] mention a lytic effect of cysteine, and Lehninger [3] has extensively studied swelling induced by 10 mM glutathione.

In the present work the swelling of isolated liver mitochondria [1] was followed by light scattering or absorbancy changes of dilute suspensions in 0.33 M sucrose containing 0.025 M tris buffer, pH 7.4. Routinely the temperature was 22–25 and 520 m μ light was used. To minimize interference by certain additions such as dyes, 775 m μ was used in some experiments. Protein was measured by the method of Lowry *et al.* [4].

Figure 1 illustrates the striking differences between the absorbancy changes occurring with low concentrations of ascorbate and those accompanying swelling induced by phosphate or β -hydroxybutyrate. Characteristically ascorbate induced swelling or lysis has a lag period averaging 20 min. This is followed by a rapid fall of the optical density to very low

* Abbreviations used in this report are: DNP for 2,4 dinitrophenol, EDTA for ethylenediaminetetraacetate, P-P for inorganic pyrophosphate, P-P-P for inorganic triphosphate, DEDTC for diethyldithiosemicarbazide, pCMB for *p*-chloromercuribenzoate, DHF for dihydroxyfumarate, DHM for dihydroxymaleate, and DHA for dehydroascorbate.

† The work in this communication was carried out with the collaboration and assistance of Francisco Guerra, Beverly Schutz, Joan Fink, Lillian Ford, Audrey Scott, and Ellen Smith.

values, much lower than the plateau seen with phosphate and substrates. An important characteristic is that ascorbate lysis occurs only with low concentrations, 0.2–1 mM being optimal. Higher concentrations lengthen

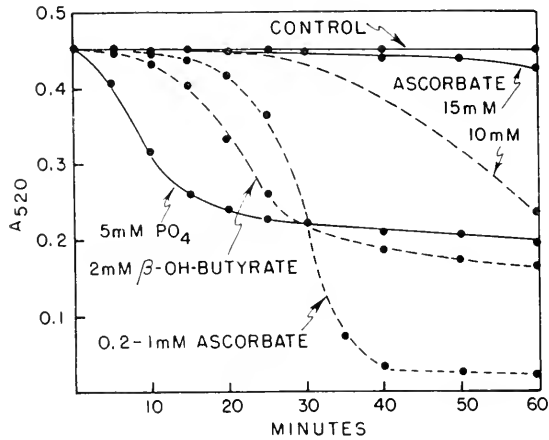


FIG. 1. Absorbancy changes at 520 $m\mu$ when dilute suspensions of liver mitochondria are treated with phosphate, β -hydroxybutyrate, or ascorbate. All additions made at zero time. Mitochondrial protein was 150 $\mu\text{g./ml}$.

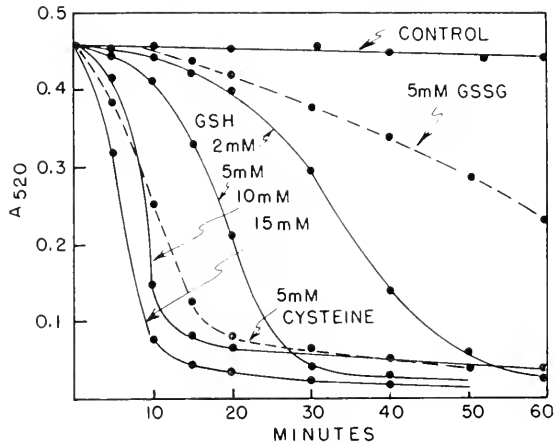


FIG. 2. Absorbancy changes due to swelling or lysis of mitochondria induced by cysteine or glutathione.

the lag period and inhibit swelling, until 15 mM prevents swelling completely for 1 to 2 hr. This has special significance, for high concentrations of ascorbate do not block phosphate or substrate-induced swelling.

Figure 2 demonstrates that reduced glutathione (GSH) and cysteine produce an absorbancy change like that seen with low concentrations of

ascorbate, but the concentrations required are considerably higher. In addition, raising the concentration shortens the lag period. If there is a high concentration which inhibits swelling it was not reached in these experiments. Oxidized glutathione (GSSG) produces a slow steady swelling, possibly the result of partial reduction. High concentrations of ascorbate do prevent glutathione induced swelling.

Ascorbate lysis is more difficult to produce as the concentration of the mitochondrial suspension is increased, and unlike phosphate-induced swelling, it is not seen at all in concentrated suspensions. While this could result from rapid exhaustion of ascorbate or O_2 , increasing ascorbate in proportion to the mass of mitochondria and thorough oxygenation have

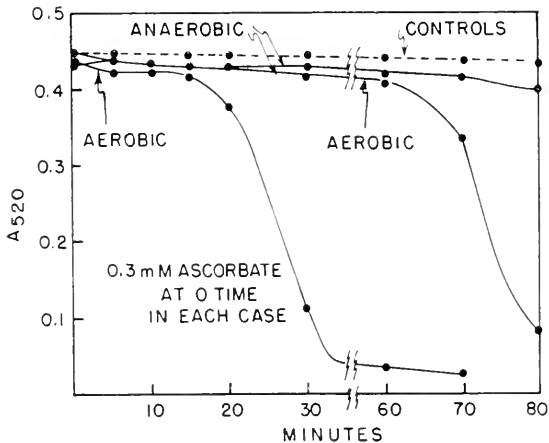


FIG. 3. Absorbancy changes due to ascorbate lysis of mitochondria are prevented as long as strict anaerobiosis is maintained. Admitting air after 50 min. results in a typical effect of ascorbate.

been only partly successful in more concentrated suspensions. Perhaps trace metal binding by the greater mass of protein is involved.

Ascorbate induced lysis of the mitochondria requires the presence of some oxygen (Fig. 3). Strict anaerobiosis will prevent lysis for at least 2 hr. If air is admitted after 1 hr., lysis occurs in the usual characteristic fashion, with the possible exception that the lag period may be a little shorter. In this requirement for oxygen, ascorbate lysis resembles swelling induced by phosphate and many other agents. While swelling with these other agents has been demonstrated to be dependent on endogenous or added substrate in nearly every case, this is not true for ascorbate. Ageing or other treatments of mitochondria which deplete endogenous substrate do not alter the response to ascorbate. Long-term ageing at 0° tends to shorten the lag period.

Because of the oxygen requirement, the effect of electron transport

chain inhibitors on ascorbate-induced lysis was investigated. These inhibitors have been shown to block the oxygen and substrate requiring swelling induced by phosphate, etc. [1, 5, 6]. Figure 4 illustrates that amytal does not block ascorbate lysis, while 2 mM NaCN, 4–6 μ M antimycin A, or 4–6 μ M SN 5949 inhibit completely. These observations suggest that inhibition of electron transport from cytochrome *b* and above prevents the ascorbate effect. Somewhat puzzling is the fact that slightly higher cyanide and antimycin concentrations are required to block ascorbate than to block the phosphate type of swelling. Moreover, 10 mM NaN_3 , which is moderately effective against phosphate, produces only slight inhibition of ascorbate and GSH induced swelling. Possibly these

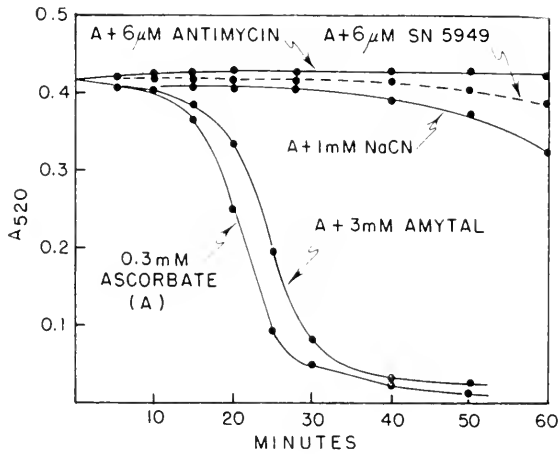


FIG. 4. Effect of electron transport chain inhibitors on the absorbance change associated with ascorbate lysis of mitochondria.

reducing agents interfere with the action of azide. Perhaps an explanation for some of the other differences will evolve from the work of Chappell and Greville [7].

Ascorbate has long been known to feed electrons into the electron transport chain via added cytochrome *c* [8, 9]. A much lower, but not insignificant oxygen consumption occurs without added cytochrome *c*. Just how much of this represents electron transfer via the cytochromes is uncertain at the moment. If ascorbate lysis depends on ascorbate oxidation (electron transfer from ascorbate), inhibition by antimycin A suggests electrons entering the electron transport chain at cytochrome *b*, ubiquinone, or lower, rather than cytochrome *c*. Figure 5 shows an experiment to test whether added cytochrome *c* would change the effect of ascorbate. It does not, but the experiment is inconclusive, as the concentration differences make it impossible for all the ascorbate to be oxidized instantaneously by

the cytochrome *c*. Some persists for a period of time, possibly feeding electrons to cytochrome *b*. It is clear that rapid transfer of electrons from ascorbate to added cytochrome *c* to O_2 via the electron transport chain, as must be occurring, is not capable of producing swelling in the presence of 15 mM ascorbate. If electron transfer through some carrier like cytochrome *b* is of special importance, one possible mechanism for the blocking action of high concentrations of ascorbate would be that cytochrome *c* is kept so completely reduced that electron transfer through cytochrome *b* is not possible.

DNP at 10^{-4} M, which blocks phosphate + substrate induced swelling under the conditions used here, does not influence the action of ascorbate

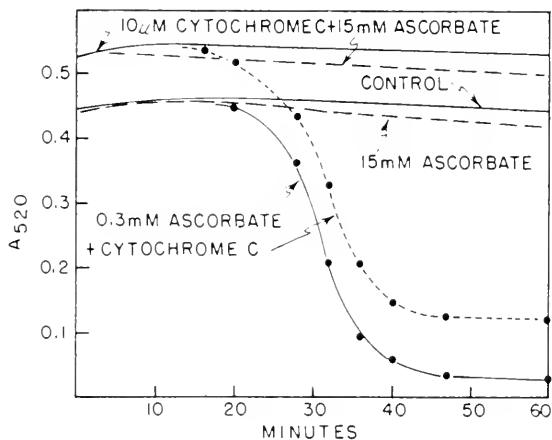


FIG. 5. Failure of added cytochrome *c* to modify the effects of either low or high concentrations of ascorbate on mitochondrial suspensions.

(Fig. 6). Lower concentrations (10^{-6} M), which hasten substrate-induced swelling, do not shorten the lag period with ascorbate.

The chelating agent EDTA (10^{-5} to 10^{-3} M) blocks ascorbate induced lysis at least as easily as it blocks virtually all other swelling inducing agents (Fig. 6). Other chelating compounds are very effective in preventing ascorbate lysis, even though they may be much less effective or ineffective in blocking phosphate type swelling. Complete inhibition of the ascorbate effect was seen with 1 mM penicillamine, 2 mM *o*-phenanthroline, 0.1 mM 8-hydroxyquinoline, 2 mM citrate, 1 mM inorganic pyrophosphate, 1 mM inorganic triphosphate, 10 mM oxalate, and 0.2 mM diethyldithiocarbamate (Fig. 6). In each case the concentration is roughly the minimum for complete inhibition lasting an hour or more. Lower concentrations cause partial block or markedly prolong the lag period.

Oxaloacetate, pyruvate, and phenylpyruvate block ascorbate (Fig. 7)

just as they do other swelling agents. Possibly they should be grouped with EDTA. As has been observed in other work Mn^{++} is considerably more effective than Mg^{++} in preserving mitochondrial structure. Because of

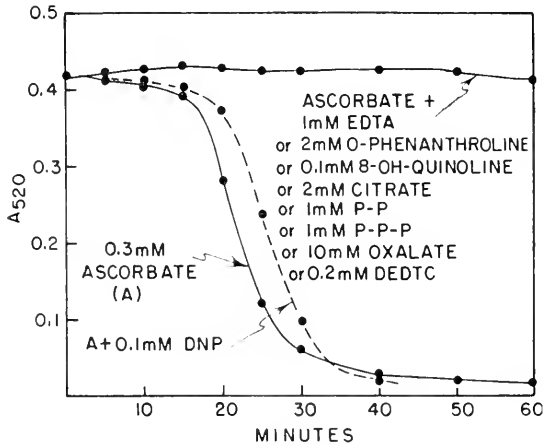


FIG. 6. Inhibition of ascorbate-induced lysis of mitochondria by metal complexing agents.

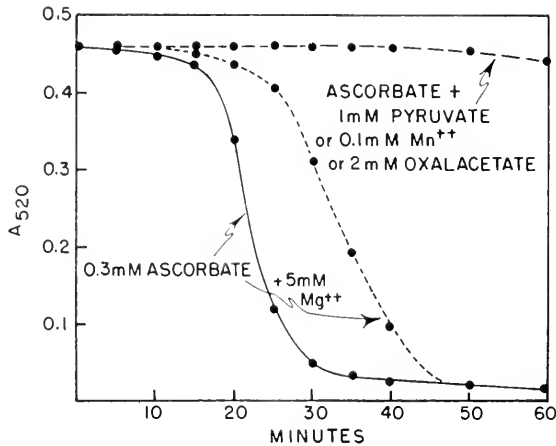


FIG. 7. Inhibition of ascorbate-induced lysis of mitochondria by Mg^{++} , Mn^{++} , pyruvate, and oxaloacetate.

the inhibitory effect of 15 mM ascorbate, several possible reducing substances were tested. Two mM nitrite has only a slight effect, but 20 mM frequently blocks completely for some time (Fig. 8). Hydroquinone and catechol completely prevent ascorbate-induced lysis in concentrations which have no effect or smaller effects on phosphate swelling. This may

well be the result of complexing with metals rather than reducing action, for quinone is even more effective than hydroquinone. At 5 and 10 mM hydroquinone or quinone alone cause a small amount of swelling of the phosphate type.

The question whether ascorbate induced lysis of mitochondria is dependent on entry of electrons into the electron transport chain cannot be answered completely just now. It appears to be dependent on some trace metal effect. For the moment we must keep in mind the fact that CN, antimycin A, and SN 5949 may act as metal complexing agents as well as electron transport chain inhibitors.

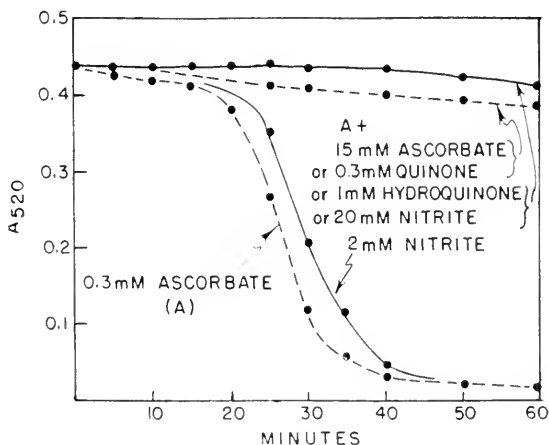


FIG. 8. Comparison of nitrite, hydroquinone, and quinone with 15 mM ascorbate as inhibitors of the lysis of mitochondria induced by 0.3 mM ascorbate.

The lytic action of low ascorbate concentrations is clearly established as a different phenomenon by experiments in which ascorbate is added after phosphate + substrate swelling is more or less complete. In Fig. 9 it may be seen that the typical ascorbate type of optical density change curve occurs after phosphate swelling. The lag is similar and the absorbancy falls to very low values. This figure also illustrates the fact that the typical lag period (usually shortened a little) occurs after the mitochondria have been at 25 for 30 min. Similar results are obtained after 60 min. This clearly indicates that ascorbate lysis is basically unchanged by ageing and is not dependent on endogenous substrate. In Fig. 10 it may be seen that the same inhibitors block ascorbate-induced lysis after phosphate swelling has occurred as with fresh mitochondria.

Because of the possibility that H_2O_2 production was involved in the metal-ascorbate induced lysis, the effects of catalase and of H_2O_2 were

tested. Figure 11 illustrates that single or multiple additions of catalase did not significantly alter the effect of ascorbate. Likewise, single or multiple additions of H_2O_2 as such, or generation of H_2O_2 by the glucose

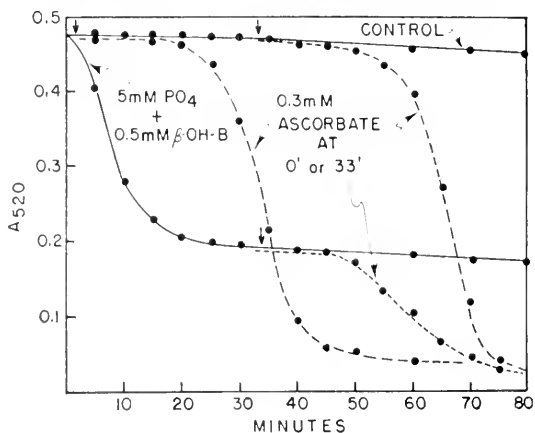


FIG. 9. Demonstration that ascorbate-induced lysis of mitochondria occurs after depletion of endogenous substrates (ascorbate added after the mitochondria were at 24' for 33 min.) and after phosphate-induced swelling has occurred (ascorbate added at 33 min.).

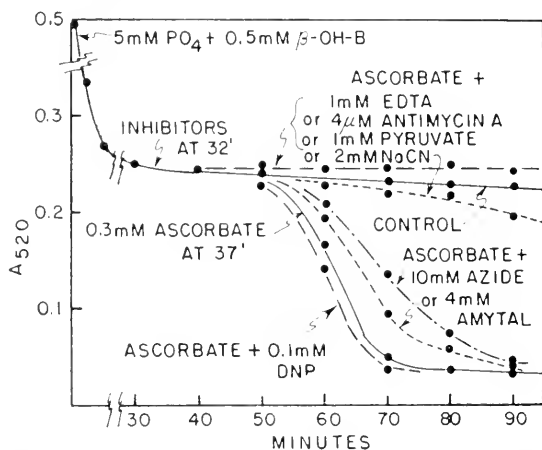


FIG. 10. Effect of various inhibitors on ascorbate-induced lysis of mitochondria after phosphate + β -hydroxybutyrate-induced swelling has already occurred.

oxidase system, did not produce an ascorbate-like effect. High concentrations of glucose oxidase tend to inhibit ascorbate lysis. This is probably due to destruction of ascorbate and to a non-specific protein effect (metal binding?). All evidence on a role for H_2O_2 is at present negative.

Because the experimental data could suggest an ascorbate-metal catalyzed oxidative change in some labile key group in the membrane, possibly not really involving the electron transport chain, we investigated

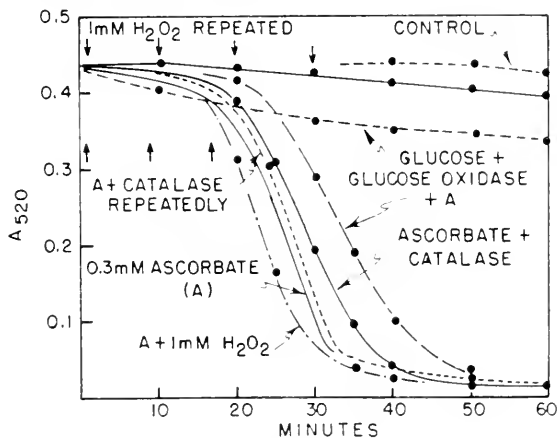


FIG. 11. Failure of added H_2O_2 , H_2O_2 -forming enzyme systems, or catalase to modify significantly ascorbate-induced lysis of mitochondria.

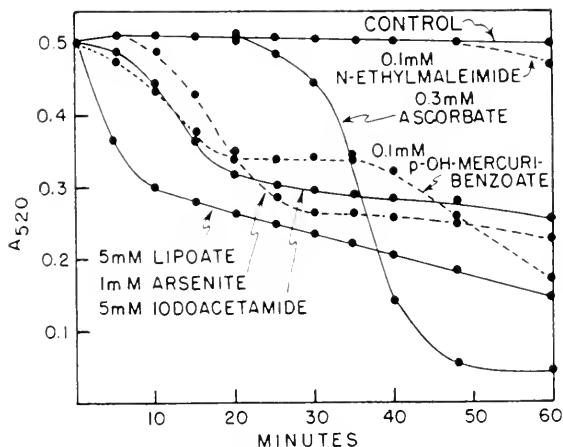


FIG. 12. Comparison of the absorbancy changes due to ascorbate lysis of mitochondria with those produced by lipoate and reagents which react with thiol and dithiol groups.

additional sulphhydryl compounds and reagents which react with thiol and dithiol groups. The swelling inducing effect of arsenite [1] and *p*-chloromercuribenzoate have been reported [10]. Carefully investigated over a wide range of concentrations, none of these compounds produced a typical ascorbate-like effect (Fig. 12). Oxidized lipoic acid, arsenite, and

iodoacetamide produce swelling, but it resembles that seen with phosphate more than that due to ascorbate. An interesting observation from these experiments is that very low *p*-hydroxymercuribenzoate concentrations produce swelling after a lag period, while 60–100 μM levels have a three-

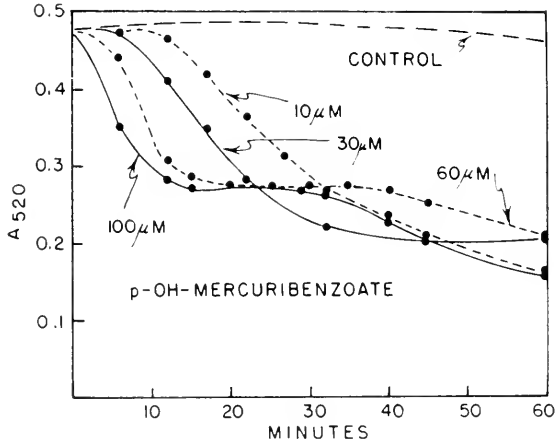


FIG. 13. Absorbancy changes due to different concentrations of *p*-hydroxymercuribenzoate. High concentrations show a three-phase curve.

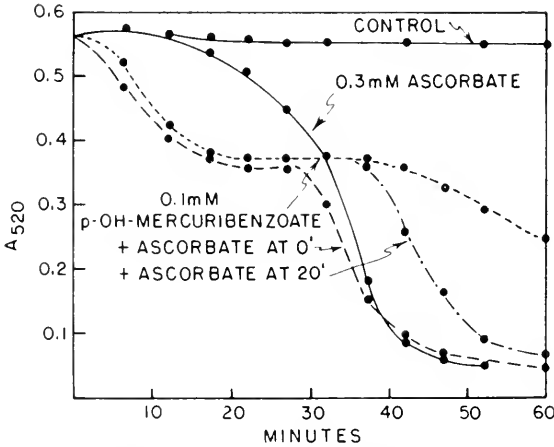


FIG. 14. Ascorbate added with or after *p*-hydroxymercuribenzoate produces fairly typical lysis.

phase effect—first a rapid fall in absorbancy, then a definite plateau for about 10 min., then a further fall (Fig. 13). This may indicate two sites of action or an immediate and a delayed effect from a single site of action. Pyruvate seems to inhibit the initial phase with little effect on the second fall.

Although reagents reacting with sulphhydryl groups did not mimic ascorbate, it was of considerable importance to determine whether reaction of these substances with the mitochondrial membrane prevented the action of ascorbate. In Fig. 14 it may be seen clearly that ascorbate lysis seems to be independent of the action of *p*-hydroxymercuribenzoate. Lysis occurs in a typical fashion whether ascorbate is added simultaneously with or 20 min. after the inhibitor. Similar data have been obtained with arsenite. These data suggest that thiol or dithiol groups may not be critical for the lytic action of ascorbate.

We have investigated the specificity of the ascorbate type of effect by testing substances structurally related to ascorbate. The ones of primary

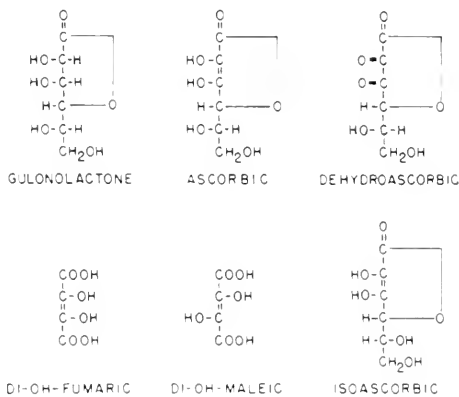


FIG. 15. Formulae for ascorbic acid and some compounds with structures related to part of the ascorbate molecule.

interest are shown in Fig. 15. Isoascorbate gives an effect identical with ascorbate (Fig. 16). In experiments followed for just 60 min., dehydroascorbate, the oxidation product of ascorbate had no effect, but longer experiments revealed that it may produce an effect after very long lag periods (60-90 min.). How much reduction occurs is unknown. The precursor of ascorbate, gulonolactone, does not induce lysis at all. Two compounds containing groups similar to the active oxido-reduction centre of ascorbate, dihydroxyfumarate and dihydroxymaleate, produce lysis which appears identical to that with ascorbate, with one important difference—the lag period is 40-50 min. instead of 15-25. The active concentrations are identical with ascorbate, and other concentrations do not give a shorter lag period.

Not only do low concentrations of dihydroxyfumarate and dihydroxymaleate act like ascorbate, high concentrations, like high ascorbate, do not cause lysis. Moreover, high concentrations of the dihydroxy acids block low concentrations of ascorbate and high concentrations of ascorbate

block low concentrations of dihydroxy acids (Fig. 17). It is also of some interest that dehydroascorbate shows steadily increasing antagonism of ascorbate action as the concentration is raised to 10 mM.

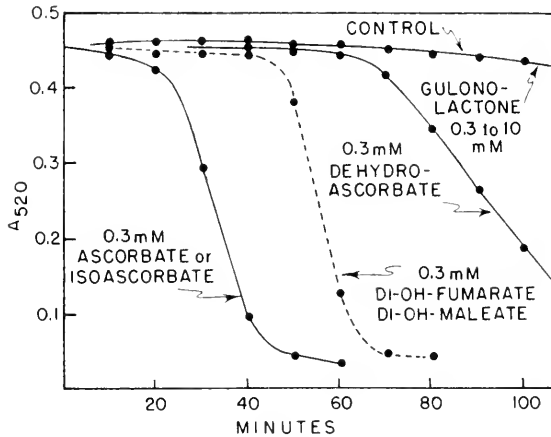


FIG. 16. Ascorbate-like lysis of mitochondria produced by certain substances with related structures. Note especially the marked differences in the lag period.

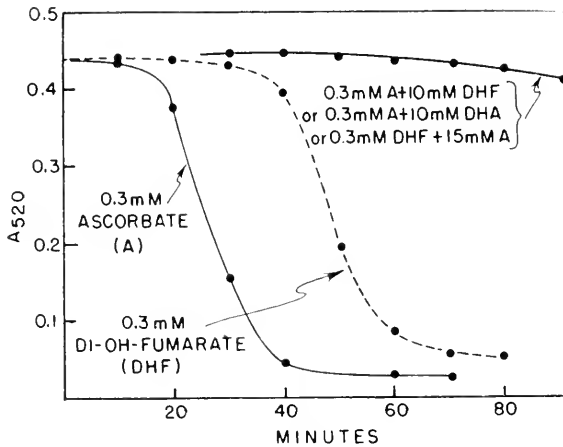


FIG. 17. Inhibition of ascorbate-induced lysis of mitochondria by high concentrations of dihydroxyfumarate (DHF) and dehydroascorbate (DHA). Inhibition of DHF-induced lysis by high concentrations of ascorbate.

More knowledge concerning what is happening during the lag period undoubtedly would tell us something about the mechanism of action of ascorbate. We have very little information on this point. In fact some titrations with indophenol raise a question as to whether ascorbate is disappearing. More experiments are needed. However, in the course of

testing various substances several striking alterations in the effect of ascorbate have been observed. Two of these are shown in Fig. 18. Five mM α -ketoglutarate usually causes a little swelling. In this experiment it caused almost none, but it drastically shortened the lag period for ascorbate lysis. Even more remarkable is the effect of 10 mM gulonolactone. Alone it never causes swelling, but it converted ascorbate-lysis into typical phosphate type swelling. Only further work can add information on these effects.

We do not know much about the mechanism of the ascorbate lysis phenomenon, but we can describe it in terms other than just light scattering

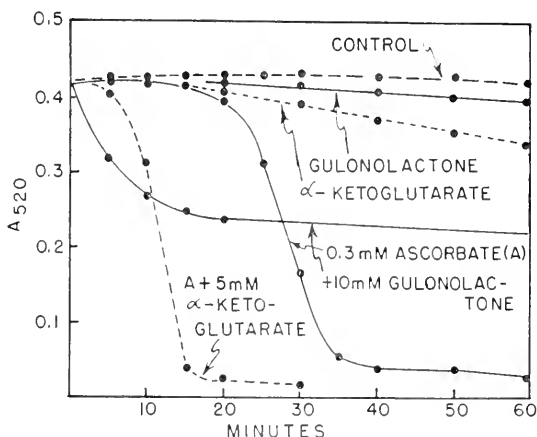


FIG. 18. Effect of α -ketoglutarate and gulonolactone on the lag period and absorbancy curve associated with ascorbate-induced lysis of mitochondria.

changes. The experiments in Fig. 19 show the distribution of protein recoveries on differential centrifugation after dilution of mitochondrial suspensions and various experimental treatments. Control suspensions, whether held at 0° or 25°, yield about 80% of the protein in the regular mitochondrial pellet at $8000 \times g$ and 15–20% in the supernatant after 1 hr. at $100\,000 \times g$. Phosphate- and succinate-produced swelling alter this distribution remarkably little. However, after treatment with ascorbate 75% of the protein appears in the “soluble” fraction, 3 to 7% in the submitochondrial particle fraction ($100\,000 \times g$ pellet). The $20\,000 \times g$ pellet contains drastically swollen and damaged mitochondria. The submitochondrial particle fraction ($100\,000 \times g$ pellet) is greatly increased by ascorbate treatment.

Electron microscopy has been used to examine the nature of the morphological changes. After phosphate-induced swelling swollen and unchanged mitochondria are clearly seen. With ascorbate treatment most

of the mitochondria disappear. The differential centrifugation yields pellets which demonstrate the disintegration of most of the mitochondria into smaller particles and soluble protein. Essentially all of the pyridine nucleotide is released into the soluble fraction during ascorbate lysis.

PROTEIN DISTRIBUTION AFTER MITOCHONDRIAL SWELLING

Treatment	Percentage recovered protein in			
	8 000 g Pellet	20 000 g Pellet	100 000 g Pellet	Super- natant
0 Control	83.0	2.6	0.5	13.7
25 Control	78.4	3.0	0.9	18.0
Ascorbate 25	10.8	10.0	7.3	72.0
Ascorbate 25	12.9	8.9	6.4	72
PO ₄ 25	83.0	1.6	0.7	14.8
Succinate 25	78.0	2.5	0.5	19.0
Ascorbate 25	12.1	8.8	2.9	76.5
PO ₄ 25	68.8	6.3	1.6	23.6
Succinate 25	71.8	8.0	0.7	19.4

Mitochondria diluted 1:25 for treatment with 0.5 mM ascorbate, or 5 mM PO₄, or 3 mM succinate, centrifuged 8 000 × g for 10 min., 20 000 × g for 20 min., 100 000 × g for 60 min.

FIG. 19. Protein distribution after mitochondrial swelling.

Figure 20 outlines some possible interrelationships between swelling inducing agents, inhibitors, and the electron transport chain. While the evidence for active electron transfer or a closely associated high energy intermediate conditioning the membrane response is strong in the case of a great many swelling-inducing substances, this question cannot be answered completely just now for ascorbate-lysis. We must determine whether inhibitors like cyanide, antimycin A, and SN 5949 act by preventing electron transport or by chelation of metal ions. There is, of course, the possibility that ascorbate-lysis is dependent on two conditions: (a) some electron transfer, and (b) an action of an ascorbate-metal complex.

The failure of dehydroascorbate (DHA) to act more like ascorbate suggests that ascorbate is not acting as a simple oxidation-reduction couple with DHA. Conceivably a half-oxidized radical [11] (rather than DHA) might be involved. Another possibility which must be given serious consideration is that ascorbate catalyzed formation of lipid peroxides [12, 13] may be responsible for disintegration of the mitochondrial membranes. Additional possibilities include alterations in lipoproteins, activation of some lytic enzyme like lecithinase, release of lysolecithin, lysoplasmalogen, or a fatty acid.

An important question not fully answered is whether ascorbate causes rupture of the membrane at one or at many points. The latter appears to be more likely, for many submitochondrial particles of small size are formed. Since ascorbate lysis occurs after swelling induced by phosphate and other agents has stopped, when the membrane permeability has greatly increased and sucrose has probably come to equilibrium internally

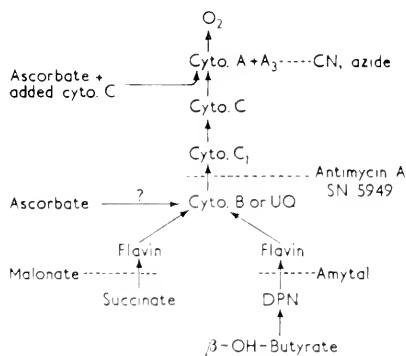


FIG. 20. Possible interrelationships between swelling inducing agents, inhibitors, and the electron transport chain.

and externally, the ascorbate effect would seem to involve rupture of links in the membrane structure and not further osmotic swelling. The action of ascorbate may give clues to key groups or links in the membrane structure. It seems unlikely that this effect is related to the vitamin role of ascorbate, but this cannot be ruled out completely.

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Discussion

WILLIAMS: May I make one general comment? I am interested in the kinetics of the swelling that you get and I have a feeling that we in the mitochondrial field should be thinking about this type of kinetics which is more familiar in the erythrocyte field where you regularly find S-shaped progress curves which represent the integral form of the Gaussian distribution of red cell fragilities. If we had better methods than just optical density measurement for following lysis then we might observe this more often. We have observed such curves in measuring the onset of choline oxidation in mitochondria which is related to the integrity of the mitochondrial membrane. It might even be useful to use them to measure the homogeneity of a population of mitochondria.

DISCHE: What was the technique of homogenization which you used in studying the distribution of different particles after ascorbate treatment, and in what medium were the mitochondria suspended when you treated them with ascorbate?

HUNTER: We used 0.33 M sucrose plus 0.025 M tris buffer pH 7.4 in all these experiments. In the protein distribution experiments the mitochondrial lysis experiment was carried out in this medium in the usual fashion and then the suspension was subjected to differential centrifugation and separated into four fractions, 8000 × g, 20 000 × g and 100 000 × g pellets plus the supernatant.

DISCHE: The distribution of fractions in the non-treated mitochondria was simply determined by centrifugation of your suspension of mitochondria?

HUNTER: We have centrifuged the suspensions at 0 and at 25 under exactly the same conditions but in the absence of ascorbate.

DISCHE: Would you not suspect that under these conditions your population of mitochondria was very inhomogeneous, because you have already got a certain distribution on your original suspension?

HUNTER: Not entirely, although it undergoes some change. Actually we think that the largest change is due to dilution. Mitochondria when diluted out as they are for experiments like this do begin to undergo changes. We have electron microscope evidence for this. If you take a mitochondrial suspension and recentrifuge it without much dilution you will get far more than 80% of the protein in the so-called mitochondrial pellet. If you dilute it out even at 0 you get some change just by that dilution, and you get only 80% of the protein in the mitochondrial fraction and an appreciable portion in the soluble fraction, which I am sure is not all a contaminating soluble protein.

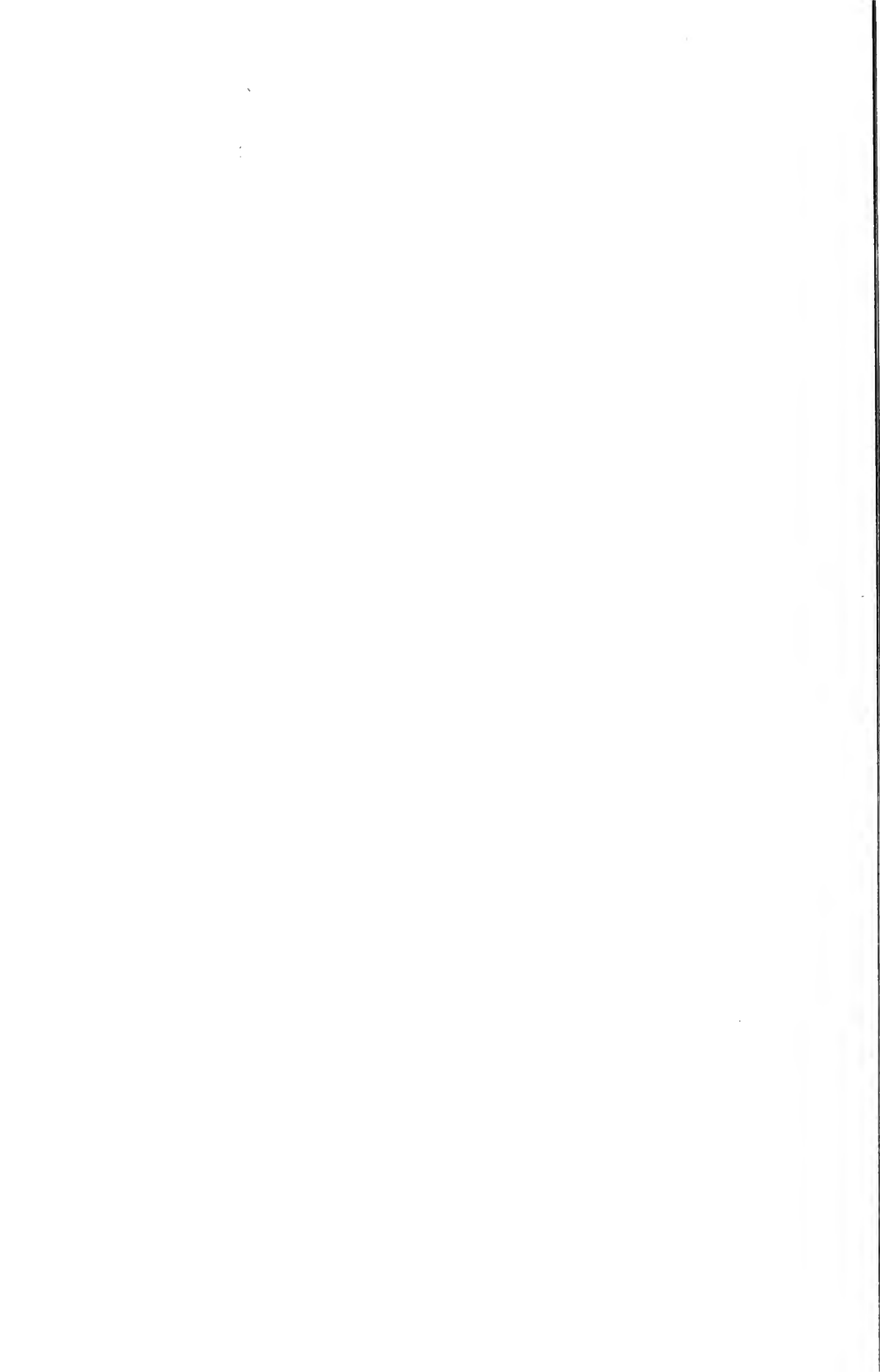
WEINBACH: Was the ascorbate effect you noted preceded by the loss of pyridine nucleotide, in other words did you examine what happened during the lag phase?

HUNTER: Within the limits of methods we use we could not say that it preceded this, but it occurs simultaneously with it.

HESS: Do you know what the fate of ascorbate is and how the kinetics are? How does it compare to the swelling action?

HUNTER: I can't answer that question exactly. We were interested in what happened to the ascorbate during the lag period. We were somewhat amazed when the preliminary experiments indicated that it was not disappearing, yet later experiments with an oxygen electrode indicated an oxygen consumption. That

might be due to stimulation of an endogenous substrate or something. These experiments do bring to mind that Ottolenghi in his experiments on lipid peroxidation by mitochondria found that ascorbate was oxidized only until the lipid was used up. Then oxidation stopped and ascorbate was not used. It probably depends on the ratio of ascorbate to mitochondria; if you use an excess of ascorbate most of it will be there when your experiment is finished.



Integrated Oxidations in Isolated Mitochondria

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It appears that, in intact mitochondria, a straightforward reduction of pyridine nucleotide by substrate, followed by reoxidation of the reduced nucleotide by the cytochrome system does not occur without complications

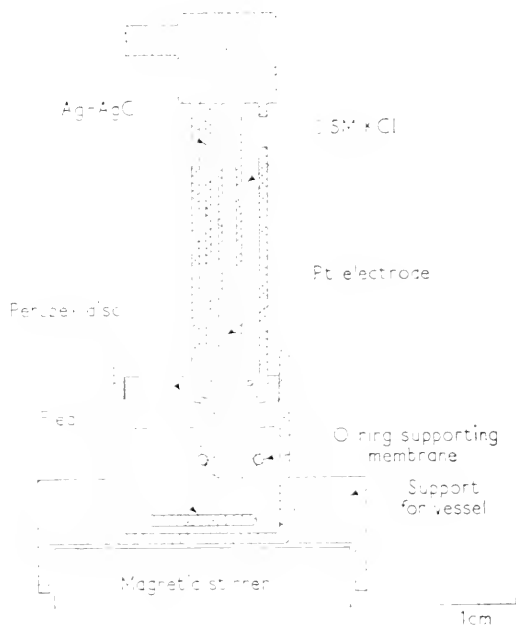


FIG. 1. The Clark oxygen electrode adapted for following mitochondrial respiration.

arising because of subsequent events at the substrate level. This paper represents an attempt to justify this statement. All the investigations reported have been performed with the Clark oxygen electrode set up as

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shown in Fig. 1 [1]. Most experiments were performed with rat liver mitochondria isolated in 0.25 M-sucrose containing 5 mM-2-amino-hydroxymethylpropane-1:3-diol-hydrochloride (tris) buffer, pH 7.4, but some results obtained with kidney mitochondria prepared in the same medium are presented.

Isocitrate oxidation

When the oxidation of isocitrate by liver mitochondria was followed in a medium containing 80 mM KCl, 6 mM MgCl₂, 15 mM phosphate and 10 mM substrate and respiration was stimulated by addition of small quantities of adenosine-diphosphate (ADP) an unusual pattern resulted

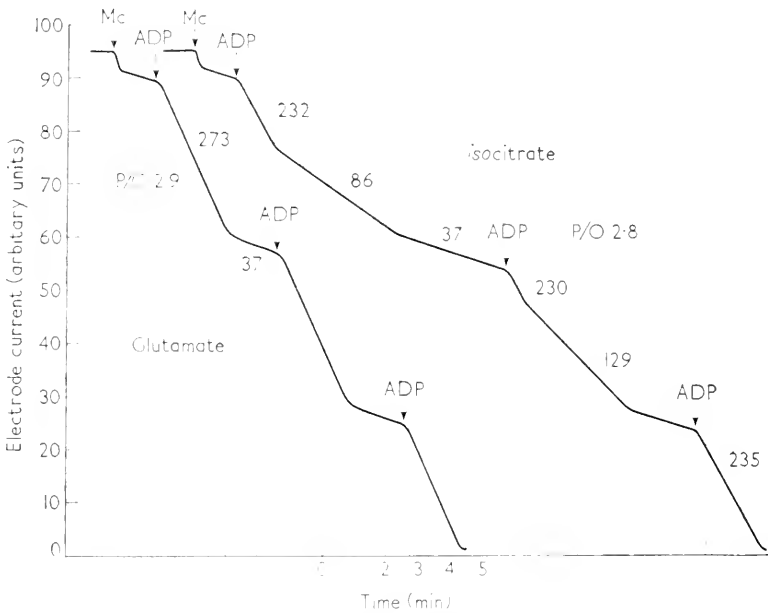


FIG. 2. Stimulation by ADP of glutamate and isocitrate oxidation in a medium containing 15 mM-phosphate. The numbers juxtaposed to the curves in this and subsequent figures represent Q_{O_2} (N) values (μ l. O₂/mg. N. hr.).

(Fig. 2). When L-glutamate, α -ketoglutarate, β -hydroxybutyrate, proline or succinate served as substrates the State 3 rate [2] was linear until nearly all the ADP had been converted into adenosine triphosphate (ATP), when the rate characteristic of State 4 ensued. With L_s(+)-isocitrate as substrate after a short period in State 3 the rate of oxidation declined and this lower rate persisted until the added ADP was exhausted. A subsequent addition of ADP after a short period in State 4 led to a further rapid rate of oxidation followed by a slower rate. However, the longer the period of

observation the less obvious was the second slower rate. When 10^{-5} to 10^{-4} M 2,4-dinitrophenol (DNP) was used to stimulate isocitrate oxidation an initial rapid rate was observed followed by a very much slower rate, which persisted for at least 15 min.

When the phosphate concentration in the medium was reduced below 5 mM, linear rates of isocitrate oxidation occurred, both when ADP and

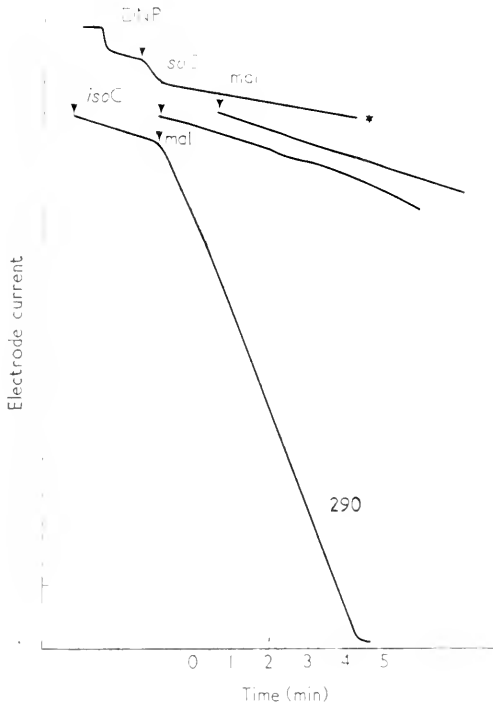


FIG. 3. The stimulation of isocitrate oxidation by malate. Three separate experiments are shown. In each case 10^{-4} M DNP was added followed 4 min. later (*) either by 1.0 mM L-malate or 10 mM L-(+)-isocitrate, or isocitrate and then malate.

DNP were used to stimulate respiration. However, even in a medium containing a low concentration of phosphate, if the mitochondria were depleted partly of their endogenous substrates by preincubating them with DNP or ADP for 5 min., added isocitrate was not oxidized at appreciable rates for many minutes. The addition of low concentrations of malate, fumarate or higher concentrations of oxaloacetate led to a marked increase in oxygen consumption. In the absence of isocitrate, and under these conditions, malate, fumarate or oxaloacetate did not produce any marked oxygen uptake (Fig. 3).

Either 5 mM-malonate or β -chlorovinylarsenious oxide ($0.5 \mu\text{g./ml.}$) severely inhibited isocitrate oxidation even in the low phosphate medium. These inhibitory effects were largely reversed by the addition of low concentrations of the dicarboxylic acids mentioned above. Half maximal effect was obtained with 2×10^{-4} M-malate. With mitochondria which had been depleted of their endogenous substrates, or in the presence of malonate or β -chlorovinylarsenious oxide, neither acetoacetate nor pyruvate together with bicarbonate were able to restore isocitrate oxidation.

These results suggest that the pyridine nucleotide reduced by the isocitrate dehydrogenase is reoxidized by coupling with the malate dehydrogenase. Neither the β -hydroxybutyrate dehydrogenase nor the malic enzyme appears to be able to do this. The scheme outlined in Fig. 4 is thought to represent the sequence of events occurring in the oxidation of isocitrate by liver mitochondria. This scheme, besides accounting for

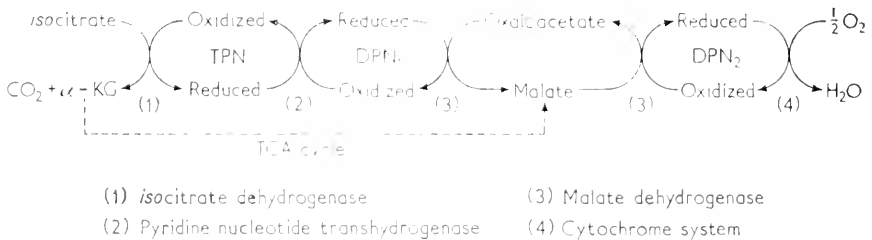


FIG. 4. The proposed pathway of isocitrate oxidation.

the experimental observations which have been given above, also takes into account the following facts: (1) there exists in liver mitochondria a triphosphopyridine nucleotide (TPN)-linked and not a diphosphopyridine nucleotide (DPN)-linked dehydrogenase [3, 4], (2) the DPN specificity of the mitochondrial malate dehydrogenase [5].

Similar results to those given above have been obtained with kidney mitochondria.

COMPARISON OF GLUTAMATE AND ISOCITRATE OXIDATION

In liver mitochondria two possible routes of glutamate oxidation are available, one involving glutamate-aspartate transaminase, the other utilizing glutamate dehydrogenase [6, 7]. The transaminase pathway is analogous to the pathway which appears to exist for isocitrate oxidation; both are coupled oxido-reductions and both involve the utilization of oxaloacetate. The former uses pyridoxal phosphate as a cofactor, the latter pyridine nucleotide. The pathway for glutamate oxidation involving the use of the dehydrogenase, leads to the reduction of pyridine nucleotide,

which in this case is directly oxidized by the cytochrome system, since, even in mitochondria depleted of their endogenous substrates, glutamate was oxidized immediately and rapidly. This was also the case when β -hydroxybutyrate, proline and malate served as substrates.

However, even when glutamate is oxidized by the dehydrogenase pathway, the rate of this reaction is intimately dependent upon the rate at which α -ketoglutarate is removed. This can be shown clearly by a study of the effects of β -chlorovinylarsenious oxide on glutamate oxidation (Fig. 5).

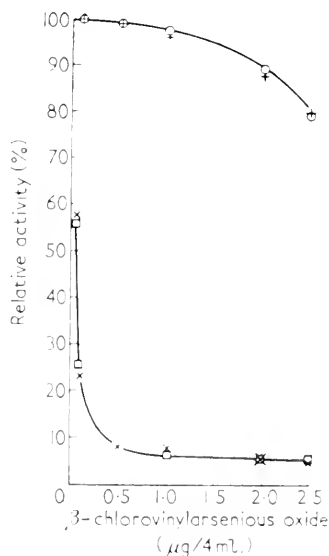


FIG. 5. The effect of β -chlorovinylarsenious oxide on ADP-stimulated oxidation of glutamate (\times — \times), α -ketoglutarate (\square — \square), succinate (+—+) and proline (o—o).

This arsenical inhibited oxidation of glutamate and α -ketoglutarate to the same extent at the same concentrations. Under identical conditions β -hydroxybutyrate, malate, proline, and succinate oxidation and the associated phosphorylation were unaffected. The inhibitory effect was readily reversed by 2:3-dimercaptopropanol (Fig. 6). The arsenical, at these concentrations, does not inhibit the glutamate dehydrogenase itself, since in disrupted mitochondria, when DPN and cytochrome *c* were added, β -chlorovinylarsenious oxide did not inhibit glutamate oxidation. In intact mitochondria it appears that glutamate oxidation cannot occur when α -ketoglutarate accumulates. Alternatively it may be concluded that the glutamate dehydrogenase is not functional in intact liver mitochondria and only serves to "spark" the oxidation of glutamate by the transaminase

pathway. The possibility must not be overlooked that glutamate dehydrogenase serves a synthetic rather than a degradative function in liver mitochondria.

The phosphate requirement for DPN-stimulated glutamate oxidation [8, 9] and, when oligomycin [10] is present, the requirement for ADP [11] are presumably reflections of the demands of the substrate level phosphorylation associated with α -ketoglutarate oxidation. These requirements have also been observed when ferricyanide acted as terminal electron acceptor.

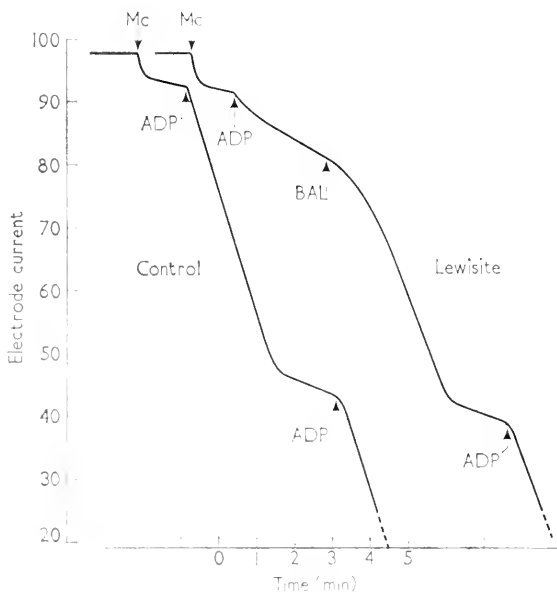


FIG. 6. The effect of 2:3-dimercaptopropanol (BAL) on the inhibition of glutamate oxidation by β -chlorovinylarsenious oxide. β -chlorovinylarsenious oxide, $0.5 \mu\text{g.}/4 \text{ ml.}$; BAL, $1 \mu\text{g.}/4 \text{ ml.}$

Similarly, isocitrate oxidation showed the same requirement for phosphate, and in the presence of oligomycin, for ADP, when respiration was stimulated by DNP. In this case it is not the accumulation of α -ketoglutarate, but the failure to produce sufficient quantity of oxaloacetate, required for the coupling process, which is responsible for the low rates of oxygen consumption. The addition of malate, in catalytic quantities, abolishes the requirement for phosphate and ADP, for isocitrate oxidation.

Succinate oxidation

In confirmation of the findings of Azzone and Ernster [12] liver mitochondria which had been pre-incubated with 2 mM arsenate and 10^{-4} M

DNP did not oxidize succinate at significant rates until ATP was added (Fig. 7). 1 mM ATP, ADP or inosine triphosphate were all equally effective. However, lower concentrations of ATP were less effective, unless oligomycin ($2.5 \mu\text{g./ml.}$) and amytal were added. Under these conditions 3 μmoles of ATP served to catalyze the oxidation of 2 μmoles of succinate, and it is apparent therefore that ATP is not required in stoichiometric amounts. Furthermore, since oligomycin inhibits the enzymes involved in oxidative

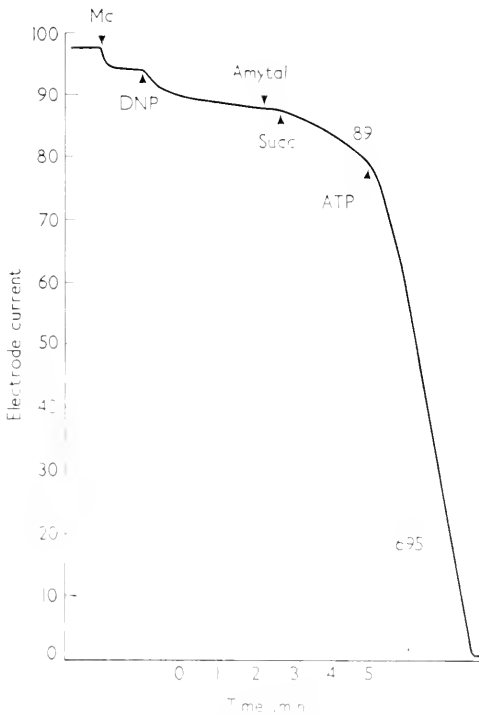


FIG. 7. Effect on succinate oxidation of preincubating liver mitochondria with DNP and arsenate (cf. Azzone and Ernster, [12]). DNP, 10^{-4} M; amytal, 1.8 mM; succinate, 10 mM; ATP, 1 mM.

phosphorylation [10] it is unlikely that ATP acts by reversing this process as suggested by Azzone and Ernster [12]. Indeed oligomycin accentuated the effect of ATP, especially at low nucleotide concentrations, presumably because this antibiotic inhibits the mitochondrial DNP-stimulated ATPase [10].

When 1.8 mM amytal, as well as arsenate and DNP, was present during the preincubation period, ATP was not required for succinate oxidation. The amytal almost entirely abolished the endogenous respiration of the liver mitochondria. With kidney mitochondria, which had an almost

immeasurably small endogenous respiration, preincubation with arsenate and DNP did not induce a requirement for ATP for succinate oxidation. However, when 0.5 mM malate was present during the preincubation period, the situation was exactly the same as it was with liver mitochondria, namely ATP was required before succinate was oxidized at significant rates and the addition of amytal at zero time, which of course prevented the oxidation of malate, abolished the requirement for ATP. It is a reasonable hypothesis therefore that when liver mitochondria are preincubated with

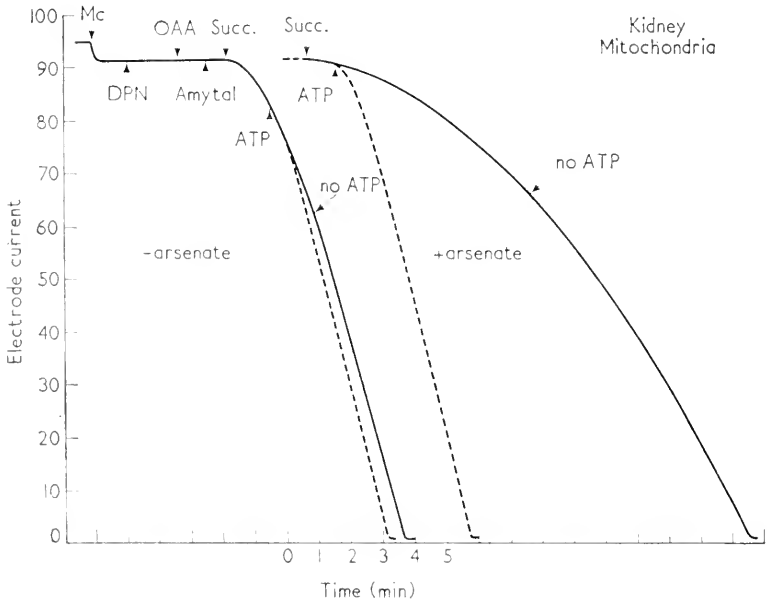


FIG. 8. Effect of arsenate and oxaloacetate on succinate oxidation by kidney mitochondria. The oxaloacetate concentration was 1 mM, other conditions as for Fig. 7. The early parts of the traces and the additions made, were the same in all cases, but are not shown for the two traces on the right.

arsenate and DNP the endogenous substrates give rise to oxaloacetate which is responsible for the inhibition of succinate oxidation. This effect can be demonstrated directly with kidney mitochondria (Fig. 8). In this case, when DNP, arsenate and oxaloacetate were added before the succinate, ATP was required for maximal rates of oxidation. Oxaloacetate had no effect in the absence of arsenate. The amount of oxaloacetate required to produce this effect was about 1 mM, which was 40–100 times greater than the amount of oxaloacetate which can be calculated to have arisen from added malate in the experiment described above. It is possible that enzymically generated oxaloacetate is more effective because it is produced

in the vicinity of the succinate dehydrogenase and that intact mitochondria are relatively impermeable to oxaloacetate.

With particulate preparations derived from liver mitochondria by lysis with phosphate and washing with KCl [13] and from kidney by the method of Slater [14], concentrations of oxaloacetate of the order of $10 \mu\text{M}$ had a profound inhibitory action on succinate oxidation. With these preparations permeability effects would be expected to be far less pronounced. However the order of addition of substrate and inhibitor had a

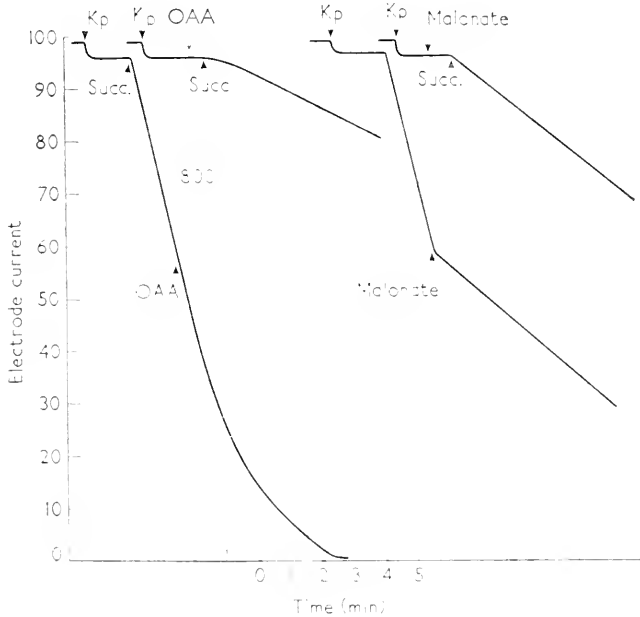


FIG. 9. Effect of oxaloacetate ($12.5 \mu\text{M}$) and malonate ($125 \mu\text{M}$) on the oxidation of succinate (10mM) by a Slater kidney preparation. The medium contained 20mM tris, pH 7.45.

marked effect on the inhibition of oxygen uptake. This is illustrated for a kidney preparation in Fig. 9. If 10 to $50 \mu\text{M}$ oxaloacetate were added before the succinate, no oxygen uptake occurred for approximately 1 min., after which the steady-state of inhibited respiration was observed. On the other hand, when the oxaloacetate was added after the succinate several minutes elapsed before the steady-state was established. In the latter case, the lower the oxaloacetate concentration the longer was the time taken before the final rate was observed. In contrast the order of addition of substrate and inhibitor was unimportant when malonate and pyrophosphate were used.

The same dependence of order of addition on the immediate effect of oxaloacetate on succinate oxidation has been observed with intact liver and kidney mitochondria. Mitochondria which had been preincubated with arsenate, DPN and amytal, oxidized succinate at rapid rates. However, if 1 mM oxaloacetate were added as little as 2 sec. before the succinate, oxygen uptake was severely and sometimes completely inhibited, whereas if the inhibitor were added after the succinate no significant effect was observed (Fig. 10).

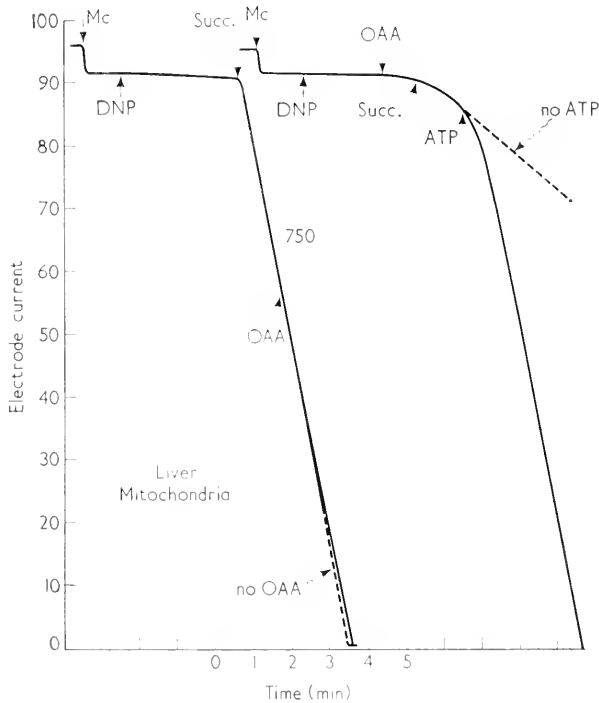


FIG. 10. Effect of adding oxaloacetate (1 mM), both before and after succinate, on the rate of oxygen uptake of liver mitochondria. 2 mM arsenate and 1.8 mM amytal were present at zero time. Other conditions as in Fig. 7.

These observations enable an explanation of the effect of Azzone and Ernster [12] to be given. Preincubation with DNP and arsenate leads to the accumulation of oxaloacetate from endogenous substrates, the keto-acid then forms a stable complex with the succinate dehydrogenase, which dissociates with difficulty. Very little can be said of how ATP reverses this inhibition; it may be that in some way ATP dissociates the dehydrogenase-oxaloacetate complex, but this is unlikely since ATP had no effect on the oxaloacetate inhibition of succinate oxidation by kidney preparations

and saline-phosphate treated liver mitochondria. An alternative hypothesis is that the ATP is required for removal of the oxaloacetate by the phosphoenolpyruvate carboxylase reaction.

Malate oxidation

EFFECT OF FLUOROMALATE

DL-3-fluoromalate is a competitive inhibitor of purified mitochondrial malate dehydrogenase; 2 mM fluoromalate causes a 99% inhibition of DPN reduction with 1 mM L-malate as substrate [5]. Malate oxidation occurs at relatively low rates in liver mitochondria, especially when DNP is used to stimulate respiration. However, when glutamate and β -chlorovinyl arsenious oxide (0.5 μ g./ml.) were used the oxaloacetate produced by the malate dehydrogenase was removed by transamination and a rapid rate of oxygen uptake resulted. The arsenical inhibited the oxidation of glutamate, as was described previously, and aspartate was shown to accumulate in the medium. In this system 5 mM fluoromalate inhibited the oxidation of 10 mM malate by more than 90%; at lower malate concentrations the inhibition was even more marked.

However, fluoromalate, even at a concentration of 10 mM, had no observable effect on two systems which are thought to involve the oxidation of malate, namely the isocitrate system (Fig. 4) and the inhibition of succinate oxidation which occurs when mitochondria are preincubated with arsenate and DNP. Both these latter systems may be thought of as "internal" and it may be that fluoromalate is unable to penetrate to them. Mitochondria behave as though they are partly impermeable to oxaloacetate and it is not inconceivable that they are also impermeable to fluoromalate.

Figure 11 is a summary of some of the findings which have been discussed and an attempt to correlate the structural relationships of the enzymes within the mitochondrion with their function.

It is apparent that the level of oxaloacetate in mitochondria can under certain conditions control the rates of glutamate, isocitrate, succinate and malate oxidation. Under conditions in which the rate of oxaloacetate production is inhibited (lewisite, malonate) glutamate and isocitrate oxidation occur at markedly reduced rates. On the other hand succinate and malate oxidation are inhibited by oxaloacetate accumulation. If these factors are suitably controlled, e.g. by providing sufficient oxaloacetate for isocitrate oxidation or by preventing the accumulation of this keto-acid in the case of malate and succinate oxidation, the rates of glutamate, isocitrate and malate (in the presence of lewisite and glutamate) occur at the same rate (360-400 μ l. O₂ mg. N hr.) when ADP is used to stimulate respiration. Succinate oxidation occurs at 50-60% greater rates. However in the

presence of 10^{-4} M DNP only glutamate and isocitrate oxidation occur at these rates; both malate (600–800 μ l. O_2 /mg. N/hr.) and succinate (up to 1600 μ l. O_2 /mg. N/hr.) oxidation occur at considerably greater rates. These results indicate that many mitochondrial oxidations are controlled by the activity of the enzymes directly involved in the synthesis of ATP and subsequent to the site of action of DNP. On the other hand β -hydroxybutyrate was oxidized at only 60% of the ADP-rate for other DNP

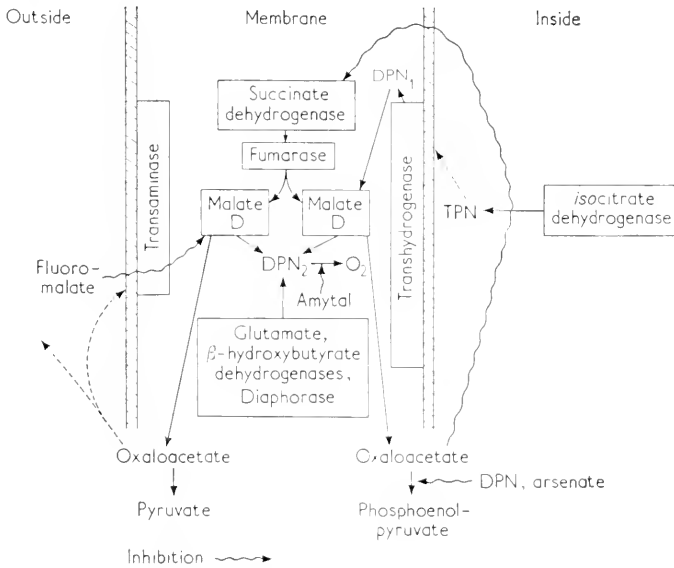


FIG. 11. Postulated spatial relationship of some mitochondrial enzymes.

linked substrates; in this case some other factor, presumably the activity of the dehydrogenase, controls the rate of oxidation.

Acknowledgment

I wish to thank Miss Freda Johnson for her expert technical assistance.

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Discussion

LOWENSTEIN: Dr. Chappell's first slide showed that isocitrate was oxidized as rapidly as glutamate. Some years ago Dr. Lardy showed that isocitrate is oxidized much more slowly than glutamate. Is this a question of the conditions that you use?

CHAPPELL: Dr. Lardy was using Warburg manometers and I am using an oxygen electrode.

LOWENSTEIN: We used an oxygen electrode and obtained the same results.

CHAPPELL: What was your level of phosphate, M 25 phosphate?

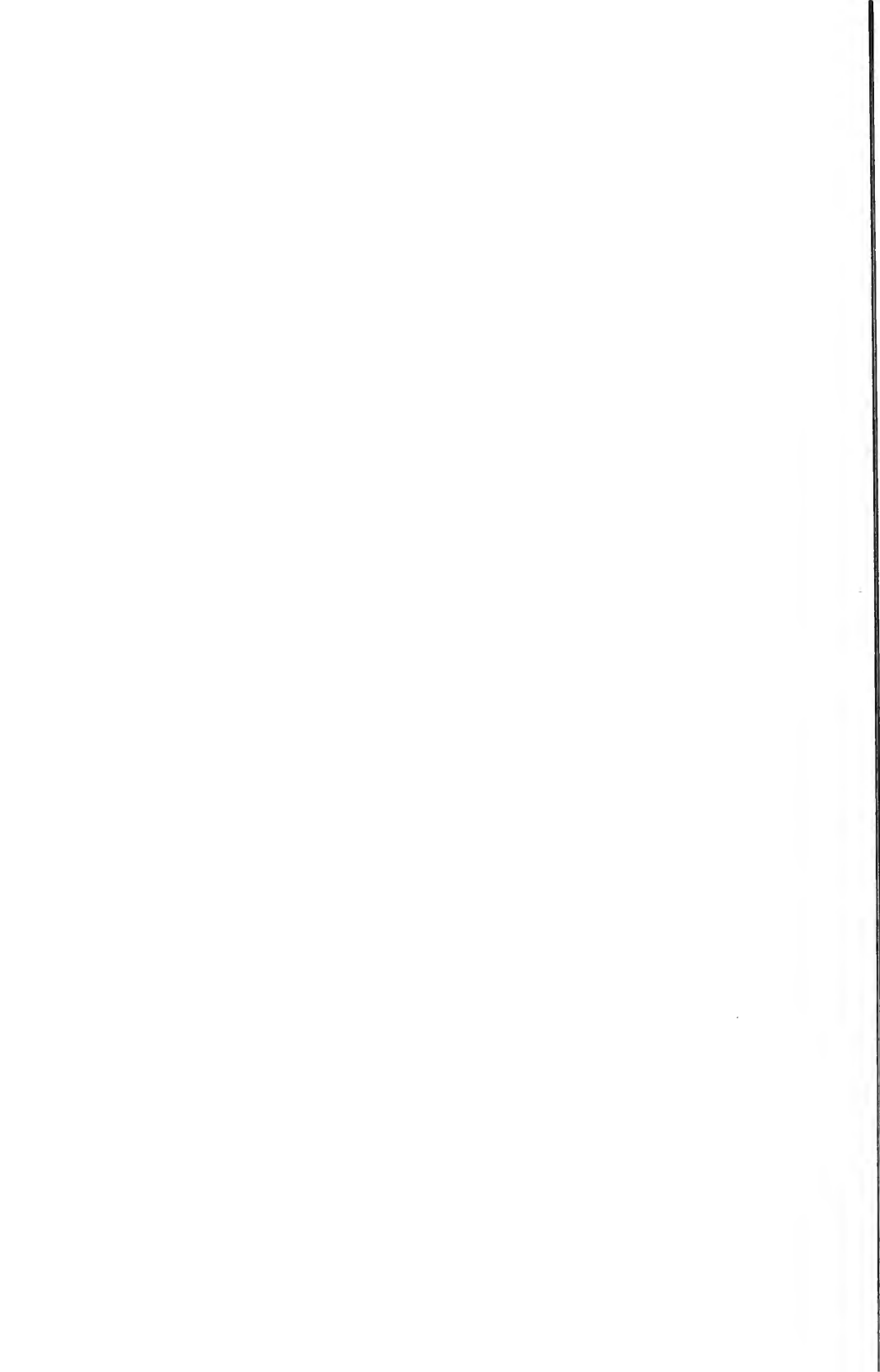
LOWENSTEIN: We used somewhat lower concentration, plus hexokinase and glucose.

CHAPPELL: M 25 phosphate is inhibitory unless you add catalytic amounts of malate. I think the point of action of the phosphate is on fumarase because at low fumarate concentrations phosphate does inhibit fumarase quite markedly, thus it is acting as malonate would, or Lewisite, and preventing the catalyst from getting into its position to catalyse more citrate oxidation.

LOWENSTEIN: You think it is the concentration of orthophosphate which is critical?

CHAPPELL: I am sure it is, you can show it very easily.

LARDY: I should like to point out that De Luca and Steenboeck have found very striking differences between the rate of oxidation of isocitrate in rats with and without vitamin D and they think that this is a controlling factor determining the levels of citrate in tissues; there is a considerable increase in the citrate content of tissues in the animals getting the normal supplement of vitamin D. I wonder whether your experiments could not be investigated using vitamin D-deficient rats to see what effects you would get.



Metabolic Control of Structural States of Mitochondria

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I would like to discuss briefly certain properties of the swelling-shrinking phenomenon as it occurs *in vitro* and perhaps in the living cell. Although many of the questions surrounding this phenomenon are unanswered, some experiments suggest directions in which explanations may lie.

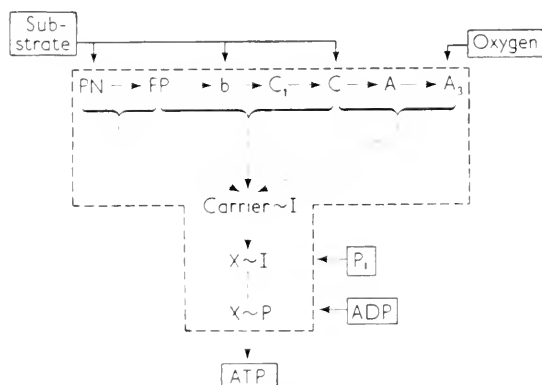


FIG. 1. Scheme for oxidative phosphorylation. The components of the mitochondrial membrane are enclosed by the dashed line.

In particular there are two points I would like to raise, both are still speculative, but it appears that their clarification may contribute to our understanding of the phenomenon at the macromolecular and cellular levels. The first is that it seems certain that changes in mitochondrial volume can be directed *in vitro* by the activity of the enzymes of the respiratory chain and oxidative phosphorylation. The system is schematized in Fig. 1. The reactions enclosed by the dashed lines represent the respiratory chain and associated enzymes of the coupling mechanism located in the membrane. This outline embodies current concepts of several laboratories [1, 2] in which it is thought that following electron transport

an energy-linked form of the oxidation-reduction carriers arises, and that this component is capable of giving rise to further intermediates which interact with inorganic phosphate and adenosine diphosphate (ADP) leading to adenosine triphosphate (ATP) synthesis. The reactants of the system are clearly substrate, which may interact at different sites, and oxygen for electron transport, and phosphate and ADP required for the synthesis of ATP. It happens that these reactants are capable of inducing characteristic changes in mitochondrial volume. The product of the process, ATP, also can control mitochondrial volume but it appears to have a rather special role. Very early in the study of the swelling-shrinking phenomenon Raaflaub [3] and Brenner-Holzach and Raaflaub [4] reported that swelling of rat liver mitochondria was retarded by ATP and also that the state of swelling was correlated with the intramitochondrial content of ATP. Dr. Lehninger and his associates [5, 6] have, of course, clearly

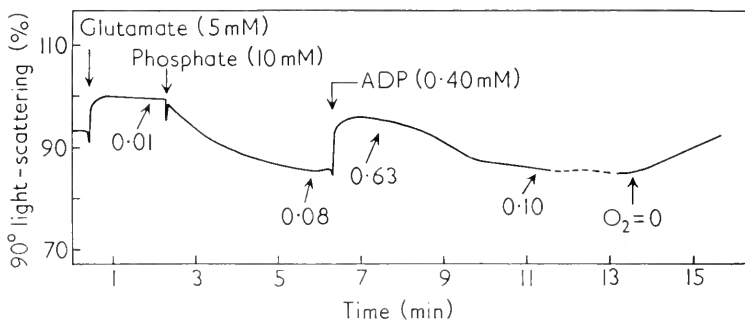


FIG. 2. Effect of the reactants of respiratory chain phosphorylation in mitochondrial swelling and shrinking.

shown that ATP acts as a potent agent to reverse swelling induced by treating mitochondria with a wide variety of the reagents, but that reversal of swelling appears to be most effective after some treatment of the mitochondria occurs which renders the membrane more permeable to this substance. Thus reversal of swelling by ATP was found to be more effective when mitochondria were suspended in potassium chloride rather than sucrose solutions. Reversal of light-scattering changes in fragmented mitochondrial membranes is also readily brought about by ATP [7]. The fact that the reactants and product of this system under appropriate circumstances can interact with the swelling-shrinking phenomenon suggests that the phenomenon is controlled by some common intermediate. An example of rapid metabolically-driven volume changes in rabbit cardiac muscle mitochondria is shown in Fig. 2. The apparatus employed was a Brice-Phoenix light-scattering photometer adapted for recording with the photomultiplier positioned at 90° to the incident beam at 546 m μ . In some

instances, kinetics of scattering changes were measured simultaneously with the utilization of oxygen by means of the vibrating platinum electrode. The electrode was placed in the cuvette and employed in a manner similar to that described by Chance and Williams [8]. Mitochondria were isolated in sucrose-Versene by the technique of Cleland and Slater [9] and suspended in a medium of 0.1 M sucrose fortified with 0.025 M "tris" buffer at pH 7.5. An increase in light-scattering indicates shrinking. Addition of reducing equivalents in the form of glutamate gave a rapid shrinkage which terminated in a steady state after a few seconds. As electron transport by tightly coupled mitochondria requires phosphate, very little respiration of glutamate was recorded at this time; this is denoted by the figure 0.01 which refers to the calculated rate of oxygen utilized in $\mu\text{M}/\text{sec}$. Adding phosphate augmented respiration sevenfold, and also initiated swelling which continued over several minutes before reaching a steady state. The reverse experiment of adding phosphate in the absence of substrate does not result in swelling. Thus phosphate is unable to induce swelling in the absence of reducing equivalents interacting with the electron transport chain. The dependence of swelling on electron transport has been widely reported on by Chappell and Greville [10] and Hunter *et al.* [11]. Initiation of phosphorylation in the mitochondrial suspension by the addition of ADP results in acceleration of respiration and a rapid shrinkage. When ADP is converted into ATP, respiration declines, and the scattering state returns to that found prior to the brief cycle of phosphorylation. Synthesis of ATP under these conditions apparently has not given rise to a net change in mitochondrial volume. When Dr. Chance and I [12] first observed this rapid reversal of swelling by ADP, and the relative lack of effectiveness of ATP under these conditions, we suggested that energy-linked intermediates may be more effective than ATP itself. Implications for the role of intermediates in the control of this phenomenon have been reported by others, for example, Ernster [13] and Lehninger and associates [5], employing different systems. Again returning to the experiment, after some lapse of time, the dissolved O_2 of the system is exhausted and at this point a reversal of swelling occurs which, if allowed to continue in this record, would have reached the level seen in the presence of ADP. This effect has been termed autonomic reversible swelling by Beechey and Holton [14]. The experiment suggests how fluctuations in the concentration of the reactants of the respiratory system are capable of controlling the state of mitochondrial volume [15, 16], as the ability of these substances to elicit changes in volume follows closely their effects upon the respiratory chain on a concentration basis. Thus the half-maximal value for activation of respiration by ADP and phosphate is about 50 μM and 1 mM respectively [17] and they have a half-maximal effect on shrinkage or swelling at the same concentrations.

Therefore it may be anticipated that a small fluctuation in the concentration of ADP would have a more extensive effect upon mitochondrial volume than a similar change in phosphate. How about the extent of the reversible volume changes? When the metabolically induced changes are calibrated by comparing them with an osmotically induced change in volume, then the calculated magnitude of the metabolic changes is of the order of 20% of their total volume [15]. It must, of course, be asked—can such changes in mitochondrial volume be induced through fluctuations in the concentrations of the reactants of the respiratory chain *in vivo*? If this is so, what effect would this have upon metabolic reactions? Perhaps in swollen or shrunken states the ability of metabolites to cross the membranes is

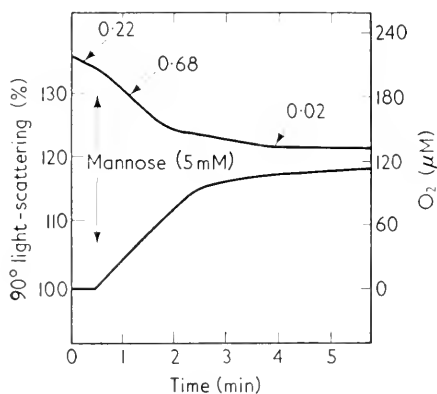


FIG. 3. Respiration and light-scattering changes in Ehrlich ascites tumour cells observed after mannose addition. The numbers above the upper trace refer to the calculated rates of oxygen consumption in $\mu\text{M}/\text{l.}/\text{sec}$.

changed. This would affect the competition which exists between cytoplasmic and mitochondrial compartments in the cell for the same metabolite. Indeed, the membrane has been implicated as a possible site for the regulation of certain effects in the intact cell such as the Pasteur and Crabtree phenomena, which involve complex interactions between different compartments in the cell. This subject was authoritatively discussed at the recent symposium on regulation of cell metabolism [18]. For this reason it seemed promising to Dr. Golder and me [19] to attempt to demonstrate light-scattering changes in ascites tumour cells following the addition of certain carbohydrates. The rapid changes in metabolism which characterize the early phases of the Crabtree effect have been probed in detail by Chance and Hess [20]. Figure 3 demonstrates a simultaneous recording of O_2 consumption and light-scattering in a suspension of these cells in Krebs-Ringer phosphate medium. Addition of mannose initiates an acceleration of respiration lasting for several minutes, which is then

followed by a strong inhibition. An increase in light-scattering also begins after mannose addition. The respiration and scattering changes are completed at almost the same time. Other hexoses such as 2-deoxyglucose and glucose give similar results. Although scattering changes have been observed which are due to tonicity changes of the cells themselves (Lucke and Parpart, [21]), rapidly penetrating carbohydrates such as mannose cause no measurable changes in cell volume as judged by direct determinations in control experiments. It was therefore proposed that the changes were of intracellular origin. Similar light-scattering or shrinkage changes are observed by ADP addition to isolated mitochondria from many sources including those of ascites tumour cells. Mitochondrial shrinkage and acceleration of respiration would be the result of the carbohydrate-induced hexokinase reaction which increases the intramitochondrial ADP level. The extensive inhibition of metabolism is believed to result from the unavailability of ATP in the cytoplasmic system. Chance and Hess [20] and Racker [22] imply that the cause is an alteration in the structure of the mitochondrial membrane. In this experiment such a change would seem disclosed by the light-scattering effect. As the production of ADP would be expected to be quite high under the conditions where scattering was increasing, these results may indicate that shrunken mitochondria *in vivo* can retard the escape of ATP synthesized by oxidative phosphorylation.

In this regard it is interesting to recall the experiments of Gamble [23] who reported increased retention of bound potassium ions by intact mitochondria under conditions of phosphorylation and, presumably, high shrinkage. Certain structural states of the membrane may favour potassium binding.

Attempts are being made to design other experiments to test the availability of ATP synthesized by oxidative phosphorylation for extramitochondrial processes. In one series of experiments Dr. Watanabe and I have made a crude reconstruction of a living muscle fibre [24]. The ATP synthesized by oxidative phosphorylation of cardiac muscle mitochondria from ADP and phosphate is made available for the isometric development of tension by a glycerinated muscle fibre. Tension development evoked by ATP alone and by ATP produced by mitochondrial phosphorylation of ADP were compared and the results are recorded in Fig. 4. Respiration of the mitochondrial suspension was traced polarographically and tension development was simultaneously recorded by use of a strain gauge transducer. It was found that tension development runs very closely with oxidative phosphorylation over a range of ADP concentrations varying between 10^{-3} and 10^{-5} M. In an experiment in the presence of ADP and mitochondria half-maximum tension was developed when the concentration of ADP was 2.5×10^{-4} M (curve A). In the absence of mitochondria with ATP only, half-maximum tension was developed

with 1.25×10^{-4} M ATP (curve B). The results show that the tension response is faster than ATP production by mitochondrial phosphorylation. Escape of ATP from the mitochondria may be the rate-limiting reaction, and this process may be connected with the extensive mitochondrial shrinkage states present.

In summary, it was shown that changes in mitochondrial volume or membrane structure are brought about by changes in the activity of the respiratory chain. Certain evidence suggests that these structural changes may lead to altered reaction rates of ATP-requiring systems which react at or near the membrane surface.

The second point which I would like to consider is the locus of the coupling mechanism involved in the swelling-shrinking phenomenon, which has been raised by some experiments we have done with *p*-chloromercuribenzoate (PCMB). Certain striking similarities between this

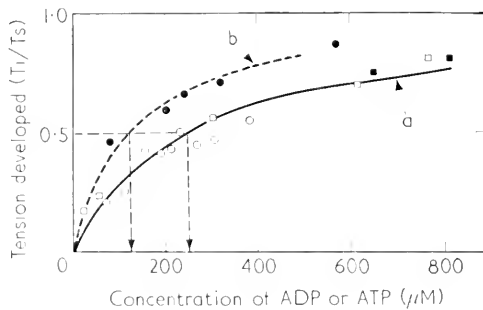


FIG. 4. Contraction of glycerol-treated fibres of rabbit psoas muscle with ATP synthesized by rabbit cardiac muscle mitochondria (a) or ATP only (b). (Courtesy of the *Journal of Biological Chemistry*.)

system and the contractile protein of muscle, myosin B, are apparent. Tapley [25] and Lehninger and Ray [26] have studied the action of PCMB on mitochondrial volume and report that it enhanced swelling. Significantly Lehninger and Ray [26] found that swelling was more rapid under aerobic than anaerobic conditions. Tapley [25] suggested that sulphhydryl groups may be important in determining mitochondrial structure. In Fig. 5 the time course is shown of the effect of $83 \mu\text{M}$ PCMB on shrinking and swelling of a suspension of cardiac mitochondria respiring different substrates. In the absence of substrate, PCMB exerts no appreciable effect upon mitochondrial shrinkage for over 2 min., and then extensive swelling occurs. When, however, PCMB is added when substrates are present it immediately initiates an enormous shrinkage. Later a reversal occurs and swelling ensues, resulting in a decrease in light-scattering similar to that in absence of substrates. PCMB-induced shrinkage is dependent on the presence of substrate and on the concentration of the SH-binding reagent. With

high concentrations of PCMB the initial shrinkage period is only transient, but at low concentrations the high shrinkage state is maintained for considerable time and reversal only occurs after long incubation times. Similar findings have been observed with uncoupling agents.

It is suggested that the period of shrinkage induced by PCMB and uncoupling agents prior to swelling is the result of their ability to interact with an enzyme of the coupling mechanism to augment adenosine triphosphatase activity recently reported by Cooper [27] through interaction with inhibitory sulphhydryl groups. However, after PCMB has been able to bind sulphhydryl groups more extensively a deformation of the macromolecular structure occurs which leads to severe swelling. Based upon the

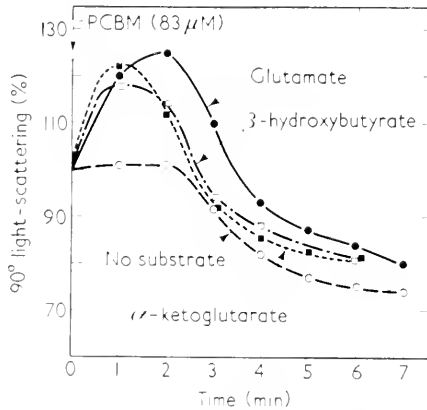


FIG. 5. Effect of *p*-chloromercuribenzoate on the shrinking-swelling phenomenon in cardiac muscle mitochondria.

current concepts of myosin B-ATPase action, it is postulated that a protein very similar to but different from the contractile muscle proteins will be isolated from mitochondrial systems as was suggested 4-5 years ago by Chappell and Perry [28].

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Discussion

LEHNINGER: These experiments carried out by Dr. Packer bring into focus one of the apparent discrepancies which can be seen in our respective approaches. Dr. Packer is interested in the physiological description of reversible, low-amplitude swelling and shrinking in tightly coupled mitochondria. Our own interest has been in dissociating the high amplitude swelling and contraction processes from all the other enzymic machinery in mitochondria which are not necessarily tightly coupled. Actually we can make mitochondria contract with ATP after respiration and phosphorylation are irreversibly lost. We have a different approach and the interesting thing is that our drastically swollen mitochondria contract with ATP but not ADP and Dr. Packer's slightly swollen mitochondria contract on the addition of ADP but not ATP. I think that his experiments point to the reason for this apparent discrepancy if I understand him right. It is now the current conception that the mitochondrial membrane *in vivo* is relatively impermeable to ATP. The internal and external nucleotides have different turnover rates. Such compartmentation has been invoked in explaining the Pasteur reaction. I think it is possible then under conditions of very drastic swelling such as we use that the permeability of the mitochondrial membrane has in fact changed, though I can hardly agree with Dr. Packer that exposure with KCl can be regarded as unphysiological. It is a more physiological substance than sucrose. In any case it is possible that for shrinking to occur ATP must penetrate inside, or that it must be generated inside from ADP. Do you believe that ADP can penetrate inside and there generate ATP which can drive the contraction?

PACKER: I think the reconstruction experiment shows that it is very difficult in isolated mitochondria to retain the ATP synthesized by oxidative phosphorylation; it almost all comes out, but when a change in permeability or when a large

swelling occurs apparently the ATP has the ability to bind on the membrane or penetrate whereas before it was ineffective. I think that ATP and ADP act through some common mechanism by influencing energy-linked intermediates. With regard to the KCl comment Jackson and Pace have shown that the half time for penetration of KCl is seconds whereas for sucrose it is hours.

LEHNINGER: If this is the case then ATP leaks very readily.

PACKER: Yes. However, this may not be so *in vivo*.

LEHNINGER: In view of what you say I am therefore a little puzzled over those explanations of the Pasteur reaction which are based on "compartmentation" of ATP.

PACKER: Well the reconstruction experiments indicate that the leakage of ATP through the membrane is rate-limiting. If we assume that in the course of isolation of mitochondria from the tissue some swelling has taken place it is reasonable that this property of the escape of ATP is now retained to a lesser extent, but still sufficiently to detect it.

HESS: If one isolates the mitochondria from glucose ascites cells then it is apparent that they take in quite a lot of ATP in comparison with mitochondria isolated from ascites tumour cells which do not contain glucose. Now the shrinkage of mitochondria seems to be associated with the retention of ATP as far as the experimental data are concerned. It is worthwhile to point out that cytologists have evidence about mechano-proteins in living cells and have found in a number of cells contractile proteins in the cytoplasm which can be readily activated by the addition of ATP. My question is: there is a certain discrepancy in the interpretation in the Crabtree effect; what is the rate-limiting material which controls rate of respiration whether phosphate or ADP? As far as I see from your data if you have low inorganic phosphate concentration, mitochondria are swollen and if you have a low ADP concentration then the mitochondria are shrunk. Now could you draw any conclusions from your experiments on this point?

PACKER: We have been interested in trying to examine the effect of phosphate on the ascites cell with respect to the scattering problem. In this connection we have tried to prepare phosphate-free ascites cells, but apparently we have not yet been able to remove sufficient phosphate to lower the endogenous respiration, so I don't feel that we have been able to put the effect of phosphate on the shrinkage phenomenon to the test.

SIEKEVITZ: I wish to recall the experiments Dr. Potter and I did in relation to the amount of ATP available in mitochondria under conditions of oxidative phosphorylation. When we added hexokinase we obtained the phosphate of the ATP as glucose 6-phosphate in the medium. Under these conditions, we found that the hexokinase does not attack the ATP inside the mitochondria but the ATP coming out. So under these conditions of active phosphorylation, I do not know whether the mitochondria are shrunken or swollen, but the ATP can come out very fast indeed.

CHANCE: Just a very short comment on the ability of ATP rapidly to enter the mitochondria. With tightly coupled mitochondria ATP can enter rapidly and at the same time cause them to swell (unpublished observations).

ESTABROOK: On the point of the comparison of rates of ATP getting out of the mitochondria and ADP going back in, one can show using the coupled hexokinase

system that the rate-limiting step with isolated mitochondria is ATP going out to hexokinase, rather than ADP going back in to the mitochondrial phosphorylating system.

PACKER: Of course very early in this study of the swelling-shrinking phenomenon Raaflaub observed that the level of swelling in the mitochondria was related to the intramitochondrial ATP level, but we may have to distinguish between ATP which is bound and unavailable and that which is free, and this may be the area where some of these discrepancies lie.

AZZONE: As far as I can understand the main discrepancy between your results and those of Dr. Lehninger is that you get shrinking when mitochondria are in State 3, that is when there is ADP in the medium and no high energy phosphate intermediate(s) can accumulate in the mitochondria; when all the ADP in the medium is transformed into ATP the mitochondria begin to accumulate high energy phosphate intermediate(s) at the same time as they begin to swell. On the contrary, in Dr. Lehninger's experiments, the mitochondria are swollen without any addition to the incubation medium, and after addition of ATP they begin to shrink. One possible explanation of this discrepancy is that in Dr. Lehninger's experiments the mitochondria are completely uncoupled. Thus it would appear that in uncoupled mitochondria external ATP is required for shrinking whereas in coupled mitochondria it is the presence of ATP and of high energy phosphate intermediate(s) inside the mitochondria which causes swelling.

Stable Structural States of Rat Heart Mitochondria

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Recent work of Packer [1, 2] has shown that isolated mitochondria of both liver and heart in dilute suspension may be induced to undergo rapidly reversible changes in light-scattering properties and that the direction of these changes is related to the relative concentrations of ADP and ATP in the reaction medium. Changes in the light-scattering properties of suspensions of mitochondria are commonly ascribed to alterations in the structure of the individual particles. This hypothesis has been adopted by Packer and is also assumed in what follows here.

Rapidly reversible changes in mitochondrial structure may also be demonstrated in the absence of external adenine nucleotide [3], but lately we have followed Packer in the use of externally added adenine nucleotide, and in experiments with heart mitochondria we have confirmed many of

TABLE I

RATIOS OF RATES OF CHANGE OF EXTINCTION OBSERVED IN A SUSPENSION OF HEART MITOCHONDRIA AT 434 $m\mu$ AND 477.5 $m\mu$ IN RESPONSE TO ADDITIONS OF PHOSPHATE AND SUCCINATE

Data calculated from Fig. 1. The values given in the last column should be compared with the theoretical ratio calculated from Rayleigh's law. The theoretical ratio for measurements at 434 $m\mu$ and 477.5 $m\mu$ where the changes of extinction are due solely to changes in the light-scattering properties of the material is 1.47 .

Experimental time (sec.)	Rate of change of extinction (τ) (m.e.u. sec.)		Ratio of rates $\frac{\tau_{434}}{\tau_{477.5}}$
	434 $m\mu$	477.5 $m\mu$	
190	1.39	0.89	1.56
230	2.21	1.50	1.47
270	1.48	1.03	1.44

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his findings. In particular, we have repeated Packer's important observation that the phosphorylating, close-coupled heart mitochondrion may be made to alternate between a stable expanded condition and a stable contracted condition according to the state of the respiratory chain (expanded

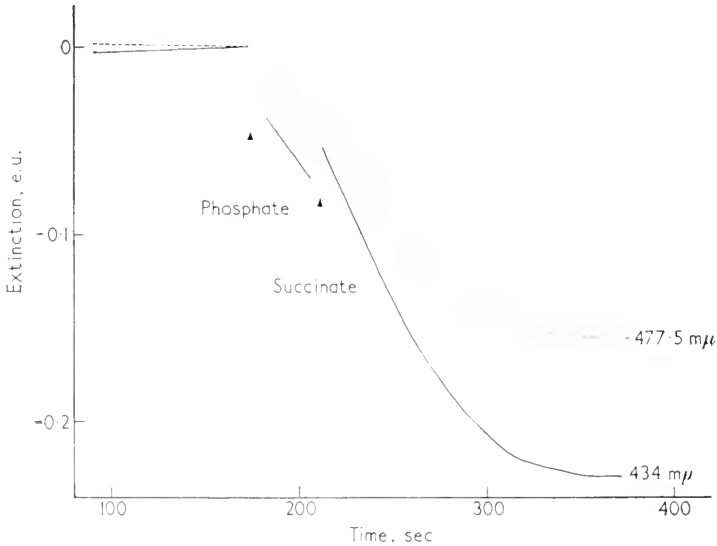


FIG. 1. Establishment of a stable expanded condition of mitochondria in response to phosphate and succinate.

Extinction changes at $434\text{ m}\mu$ and at $477.5\text{ m}\mu$ were measured simultaneously by the method of Holton [10] using a rectangular glass cell with clear walls, surrounded on three sides with polished metal plates and maintained at a constant temperature of 18.8° . Rat heart mitochondria (sarcosomes) were isolated as described previously [11] and were diluted from time 0 into a medium containing 0.28 M sucrose, 0.01 M disodium potassium ethylenediaminetetraacetate, pH 7.4 , to give 50 ml. of a reaction mixture containing 0.51 mg. biuret protein per ml. At time 177 sec. the optical recording was interrupted and 0.5 ml. of 0.5 M potassium phosphate buffer, pH 7.4 , was rapidly stirred into the suspension. At time 210 sec. 0.5 ml. of 0.4 M potassium succinate, pH 7.4 , was added in a similar way. Final concentrations were: phosphate, 4.9 mM. ; succinate, 3.9 mM.

Measurements of the rates of change of extinction observed at the two wavelengths studied yielded the ratios of rates given in Table I. These values show reasonable agreement with the ratio calculated from Rayleigh's law of light-scattering. This constitutes evidence that the progressive changes of extinction illustrated above were caused by changes in the light-scattering properties of the mitochondria.

condition with external nucleotide as ATP, respiratory chain in state 4; contracted condition with external nucleotide as ADP, respiratory chain in state 3, following the nomenclature of Chance and Williams [4]).

More recently we have attempted to answer by experiment two questions concerning the establishment of stable structural states in these

mitochondria. The first is as follows. 1. *Are there only two stable structural states or is it possible to demonstrate a series of intermediate stable states lying between the fully expanded and the fully contracted conditions?*

The second of these alternatives appears to be correct. Rat heart mitochondria may be brought to a stable expanded configuration by a short

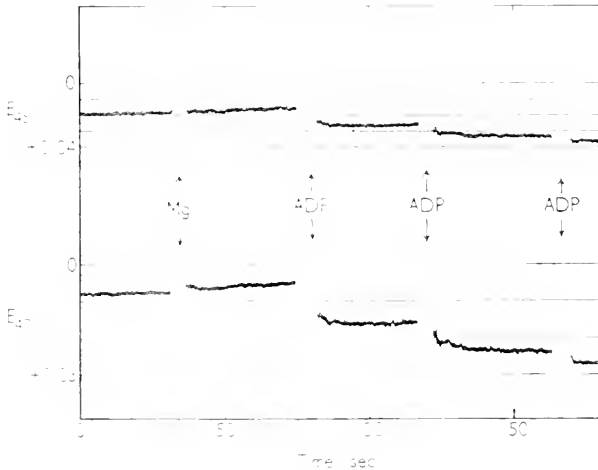


FIG. 2. Data illustrating (a) the absence of a discernible effect on mitochondrial structure when magnesium chloride is added to a suspension of heart mitochondria which have been brought to a stable expanded condition with phosphate and succinate; and (b) part of a "shrinkage titration" showing the establishment of a series of steady structural states of mitochondria with increasing concentrations of ADP.

Extinction changes were recorded and rat heart mitochondria were isolated as described in the legend to Fig. 1. Only two of the three simultaneous recordings are shown. Downward deflection of the traces indicates increase of extinction. Horizontal lines have been drawn at intervals of 0.01 extinction units. Temperature 20.9°.

9 min. before the beginning of the above record a concentrated suspension of freshly isolated mitochondria was diluted into 0.32 M sucrose, pH 7.4, to give 48 ml. of a reaction mixture containing 0.21 mg. biuret protein per ml. Phosphate buffer, pH 7.4 and potassium succinate, pH 7.4 were then added to give final concentrations: phosphate, 5.1 mM; succinate, 4.1 mM. Extinction changes similar to those shown in Fig. 1 were then observed, indicating swelling of the mitochondria. A stable expanded condition was finally established and is indicated above by the steady level of the traces at the beginning of the record. At the point marked Mg the recording was interrupted and 0.5 ml. of 0.09 M magnesium chloride was rapidly stirred into the suspension (final concentration 0.02 mM). The small decrease of extinction which followed was attributable to the effect of dilution. At the points marked ADP 0.05 ml. of 0.02 M ADP in 0.32 M sucrose was added in the same way. Three further additions of ADP were made at intervals immediately following the above record. The optical effects of these later additions are not shown above but are included in Fig. 3 together with the above data.

incubation with phosphate and succinate (Fig. 1). Addition of magnesium chloride does not alter this configuration (Fig. 2), but it activates a powerful ATPase [5] which can hydrolyze newly synthesized ATP at least as

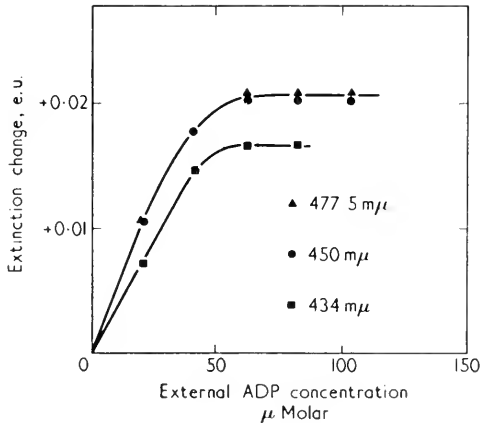


FIG. 3. Relationship between the concentration of externally added ADP and the resultant extinction changes measured simultaneously at three separate wavelengths in a suspension of heart mitochondria in the presence of magnesium ions. Data of Fig. 2 graphed, with addition of results relating to later additions of ADP and also from simultaneous observations at 434 mμ.

The changes of extinction caused by ADP clearly did not obey Rayleigh's law, since for any given concentration of ADP they did not decrease regularly with increasing wavelength of observation. The data suggests that a minor part of the extinction changes observed was due to an alteration in the extinction due to pigments as ADP was added. They are consistent with the hypothesis that the states of oxidation of both cytochrome *b* and flavoprotein were moved in the direction of oxidation by addition of ADP, an effect to be expected from the work of Chance and Baltscheffsky [12]. If it is assumed that there was no contribution of pigments to the extinction changes recorded at 450 mμ, it is possible to calculate for the other two wavelengths of observation values of the ratio

$$\frac{\text{extinction change due to light scattering}}{\text{extinction change due to pigment}}$$

For observations at both 434 mμ and 477.5 mμ the above data give a value of 3.4 for the above ratio. This value may be compared with that from the work of Chance and Packer [13], who added ADP to a suspension of rat heart sarcosomes and deduced a value of about 4 for the same ratio. (In their work the extinction change due to scattering was equated to that observed at 443 mμ and the extinction change due to pigment was equated to the difference between the changes observed at 430 mμ and at 443 mμ.)

fast as the respiratory chain, oxidizing succinate, can produce it. Under these conditions added ADP is maintained at the concentration at which it has been added while the processes of oxidative phosphorylation continue normally. Figures 2 and 3 illustrate the optical effects of successive

additions of small amounts of ADP to expanded mitochondria with magnesium present. A succession of stable structural states is established with increasing concentrations of ADP until an equilibrium contracted condition is reached.

Parallel measurements of respiration rates carried out at the same time as the spectrophotometric observations with the same preparation of mitochondria under identical experimental conditions showed that the mitochondria in their expanded condition exhibited respiratory control [6] with ADP temporarily accelerating their respiration in a characteristic way. This fact suggested a second question. 2. *Is the influence of ADP concentration on structural change quantitatively similar to its effect on respiration rate?*

We have found that the two relationships are quantitatively very similar. The results given in Fig. 2 are repeated in a different form in Fig. 3 in order to show the relation between the total concentration of added ADP and the total change of extinction caused by its addition. Half-maximal shrinkage was effected at a concentration of $18 \mu\text{M}$ ADP. Respiration measurements show that half-maximal acceleration of respiration is brought about at approximately the same concentration of ADP (e.g. $26 \mu\text{M}$ for pigeon breast muscle mitochondria [7]).

The hypothesis that the respiration rate of mitochondria *in vivo* is controlled by the concentration of ADP in the cytoplasm has been well ventilated recently [8]. Both the work of Packer and his associates and the above experiments emphasize that reversible alterations in mitochondrial structure probably represent a feature of the same control mechanism. They provide evidence supporting the suggestion of Ernster [9] that "a reversible labilization of the mitochondrial structure may constitute a physiological principle of metabolic control."

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Discussion

KLINGENBERG: May I mention that the phenomenon of reversible swelling was also reported some years ago by us for flight muscle mitochondria. These show, with glycerolphosphate as substrate, a very pronounced reversible swelling which can be related to respiratory control and to changes in the redox state of respiratory components; in further unpublished studies we noted that with pyruvate plus malate which are very effective respiratory substrates for mitochondria practically no reversible swelling could be observed. Sometimes even contraction of the mitochondria can be observed. Studies on the internal adenine nucleotide content showed that the ATP to ADP ratio in mitochondria is the same whether one has glycerolphosphate or pyruvate plus malate there. The difference in other nucleotides can only be seen in the reduction of DPN or in the reduction of flavin nucleotide, the glycerolphosphate reduces the DPN to a higher extent and the flavin also, whereas pyruvate plus malate do not reduce the DPN or the flavin nucleotide to an appreciable extent.

HOLTON: Am I right in interpreting your feeling that the reactions important in controlling structural state are at the flavin end and that they are not related directly to the ATP-ADP equilibria inside the mitochondria.

KLINGENBERG: Yes.

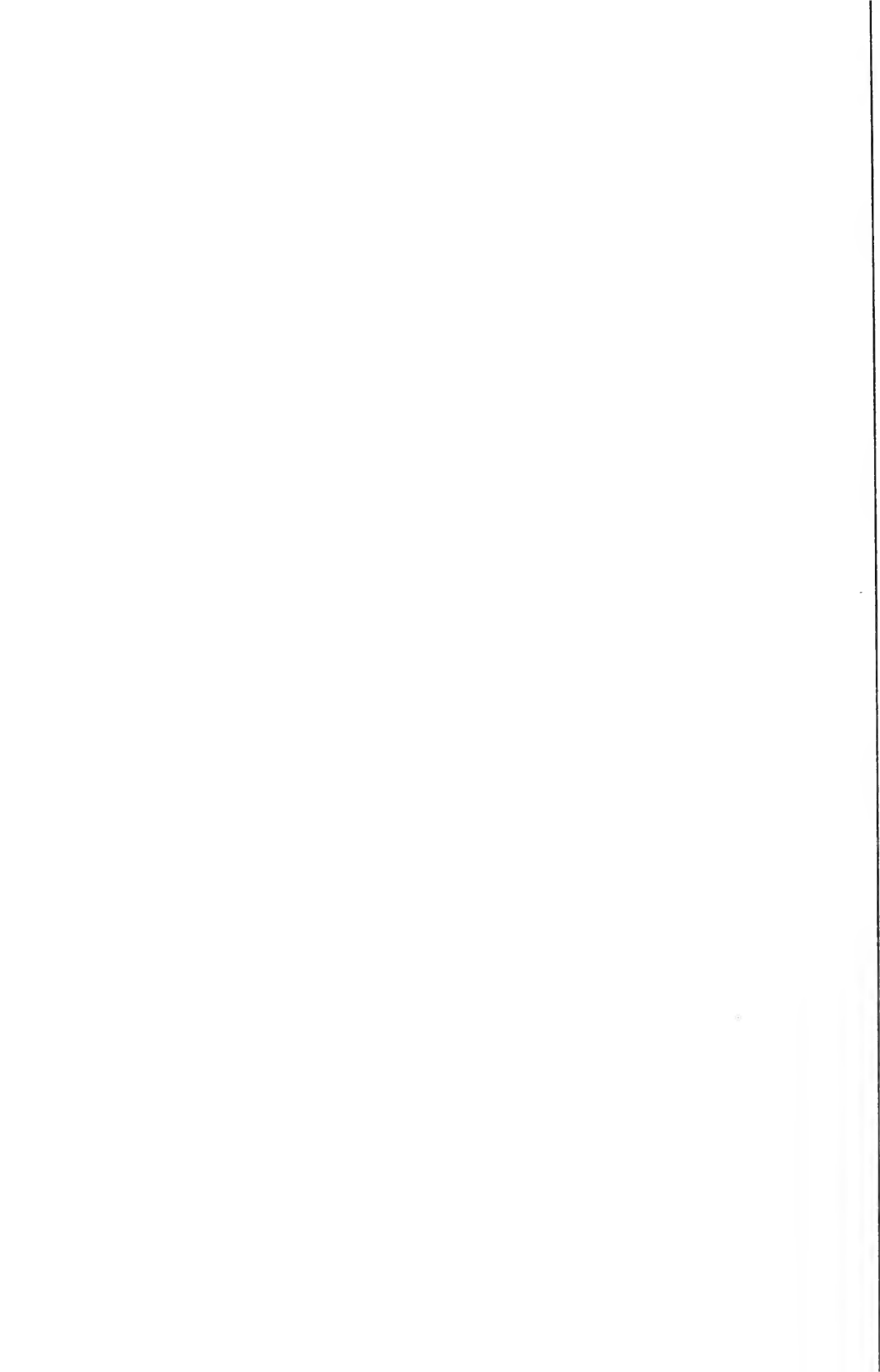
MITCHELL: You say, Dr. Holton, that this kind of swelling and shrinking, the reversible kind, is quite different from the kind studied by Dr. Lehninger. Are you in fact studying the same membrane? There are two membranes here. Are you quite sure that both these cases of swelling and shrinking are due to effects on the same membrane?

HOLTON: I would say that the fact that the mitochondria that Dr. Lehninger studies are a good deal further from the native state than ours is consistent with your idea, since the reversible phenomena that both we and Dr. Packer study don't last long. I suppose it might well be a more intact particle which shows the reversible phenomena and one which has got holes in it which shows the other.

LEHNINGER: I would just like to add that occurrence of two different morphological types of swelling could be a plausible resolution of the apparent differences in the properties. It should be made clear that there is no disagreement as to the observations. We have confirmed the effect of ADP on a tightly coupled system. On the other hand Dr. Holton has confirmed our findings that ATP is specific for shrinking drastically swollen mitochondria. As I pointed out in one of my slides there are at least two different ways in which mitochondria can swell and we have suggested in fact that it may be the outer membrane which is the ATP-activated structure. On the other hand damage to the membranes on drastic swelling could produce a difference in access of ATP and ADP, but it seems unlikely that a strict ADP specificity will change to a strict ATP specificity on damaging a structure. I would like to ask Dr. Holton a question which I think will contrast the different

kinds of systems we are studying. In the more drastic swelling we study, the amplitude of the cycle is large. There is a two or three-fold increase in volume. I would like to know whether Dr. Holton can give an estimate of the per cent difference in mitochondrial volume between his two stable states?

HOLTON: No, I am afraid that we have no data on which to base such an estimate, but perhaps a rough comparison of the magnitudes of the extinction changes we are observing in our respective systems would be helpful. I suspect that you use a 10-mm. cell and a concentration of mitochondria of perhaps 0.5 mg./2 ml. of protein. Is that reasonable? With this arrangement you get changes of about one extinction unit. Our reversible changes would not be discernible at this optical thickness and concentration. We use 0.2 mg. ml. and a 60 mm. cell where the sides are mirrored, and then we observe a total change for reversible swelling of about 0.02 extinction units. You see that there are several degrees of magnitude between the amount of extinction change which are seen under your conditions and ours.



Solubilization and Properties of the DPNH Dehydrogenase of the Respiratory Chain*

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Although the work of our laboratory for the past few years has been, in the main, concerned with the systematic isolation and detailed study of the various dehydrogenases which are structural and functional components of the respiratory chain, until recently we have not attempted to isolate one of the most interesting enzymes in this group, the respiratory chain-linked DPNH dehydrogenase. One reason for this was the large number of preparations of mitochondrial origin described in the literature which are capable of oxidizing DPNH under suitable conditions. A closer study of the relevant literature reveals, however, that few of these preparations have been well characterized; even fewer could be ascribed a definite function in cellular metabolism, and no soluble, purified preparations could be assuredly identified with the enzyme which links the oxidation of DPNH to the respiratory chain.

Among animal tissues heart mitochondria appear to have been most intensively studied with respect to DPNH oxidation. Limiting this discussion to soluble enzymes, free from respiratory chain components, there have been two relatively well-defined preparations isolated from heart mitochondria: Straub's diaphorase [1] and Mahler's DPNH cytochrome reductase [2]. Both of these enzymes have been thought, one time or another, to be artifacts of isolation, a view based on the harsh methods employed in their isolation. As to Mahler's enzyme, the group at the Enzyme Institute still believes that it is an artifact [3], since the peculiar properties of its flavin group may be reproduced by applying the alcohol treatment used in its isolation to other preparations, although Massey has produced considerable evidence to indicate that the enzyme in fact pre-exists in mitochondria [4]. In either event there is little in its properties that would suggest that it is the flavoprotein component of the DPNH oxidase chain.

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As to diaphorase, Massey's work [5, 6] has clearly demonstrated that it is part of the α -ketoglutarate oxidase complex and, as such, it is concerned with the reduction of DPN, not the oxidation of DPNH. More recently, Ziegler and colleagues [3] have reported the isolation of a lipid-bound DPNH dehydrogenase of very high molecular weight and lipid content and with very interesting properties, and King and Howard reported the extraction of DPNH dehydrogenase from heart muscle mince by treatment with phospholipase [7]. Regarding the former preparation, it remains to be seen whether its high lipid content represents a functional component or an impurity. In either case, we felt that it was desirable to isolate the dehydrogenase without recourse to organic solvents, bile salts, or other harsh treatments and in a lipid-free form, so that, as in the case of succinic dehydrogenase, the properties of the protein could be adequately characterized. The possible relation of King and Howard's soluble preparation to the enzyme I shall describe will be discussed later on in this paper.

Our initial work on this problem was concerned with the linkage of DPNH dehydrogenase to the respiratory chain. It has been known for over five years that, although succinic and DPNH dehydrogenases operate via a common respiratory chain and are interlinked at the oxidation level of cytochrome *b*, methods which solubilize succinic dehydrogenase from respiratory chain preparations [8] fail to extract DPNH dehydrogenase. Thus, superficially, the bonds holding these two closely related enzymes to the electron transport system appear to be quite different. During the isolation of α -glycerophosphate dehydrogenase from brain mitochondria a few years ago [9], we found that the incubation of brain mitochondria with phospholipase A resulted not only in the extraction of α -glycerophosphate dehydrogenase in soluble form but also of considerable DPNH dehydrogenase activity. Following this initial lead, with Drs. Minakami and Ringler, we decided to undertake its isolation and characterization [10].

Two problems faced us at the outset: the choice of starting material and the assay. In our work on other mitochondrial dehydrogenases it has been shown that these are the paramount factors deciding the success or failure of the isolation. As to starting material, we did not wish to use heart muscle mince or even mitochondria, since, besides the enzyme we were after, they were bound to contain other DPNH dehydrogenases, such as the Straub diaphorase, possibly Mahler's reductase, and any DPNH dehydrogenases arising from microsomal contamination. We decided, therefore, to use the particulate DPNH oxidase (ETP) preparation of Crane, Glenn, and Green [11], since this has been reported to be a purified form of the DPNH dehydrogenase linked to the cytochrome chain, free from numerous contaminating enzymes, particularly diaphorase, in which the oxidation of DPNH is completely antimycin and amytal-sensitive [11]. Thus any soluble preparation isolated from it would be

reasonably certain to represent the flavoprotein responsible for DPNH oxidation in mitochondria.

Considerable effort was expended on elaborating a reliable assay method for the enzyme. Early in this work it became apparent that, contrary to general impression, the assay of DPNH dehydrogenase in mitochondria or in particles such as ETP is a relatively difficult task. Among electron acceptors to be employed phenazine methosulphate was eliminated because of its rapid non-enzymic reaction with DPNH, quinones, such as menadione, because of their failure to react with the isolated dehydrogenase at significant rates, and 2,6-dichlorophenol-indophenol because of relatively high blanks and the great dependence of the measured activity on dye concentration characteristic of this oxidant.

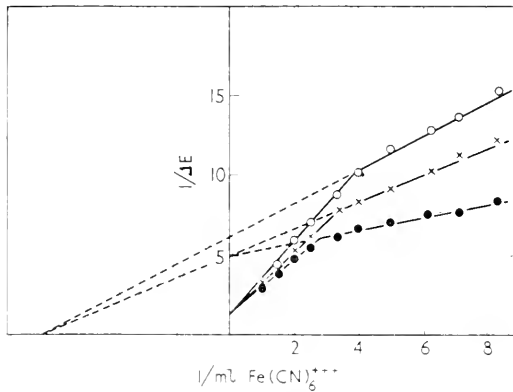


FIG. 1. Ferricyanide assay of particulate DPNH dehydrogenase in the presence of 6×10^{-4} M DPNH. The assays were performed in the presence of 120 μ moles phosphate, pH 7.4, 1.8 μ moles DPNH, DPNH oxidase, and quantities of 0.01 M ferricyanide as indicated, in a total volume of 3 ml. The determinations were made at 30° using a recording spectrophotometer and a 30 to 60 sec. total reaction time. The reduction of ferricyanide was followed at various wave lengths and corrected to E_{120} . X—X, assay without further additions, O—O, in the presence of 2×10^{-7} M antimycin A, ●—●, in the presence of 10^{-3} M cyanide.

A suitable method was eventually elaborated which is based on the spectrophotometric measurement of the initial rate (15 or 30 sec.) of reduction of ferricyanide [12]. The application of this method to heart mitochondria or to particles derived therefrom, such as ETP, entails several problems, some of which are illustrated in Fig. 1. This figure is a Line-weaver-Burk plot of the variation of measured activity with ferricyanide concentration in an ETP preparation at moderately high (6×10^{-4} M) initial substrate concentration. In the absence of inhibitors (crosses) a definite break is seen in the curve relating reciprocal activity to reciprocal ferricyanide concentration. The reason for this is that ferricyanide has two

reaction sites in the DPNH-oxidase chain of heart mitochondria, the flavoprotein and cytochrome *c*, just as in the succinoxidase system [13, 14]. The relatively flat part of the curve represents primarily the reaction with cytochrome *c*, since the apparent K_M of the *flavoprotein* for ferricyanide is much larger than that of the cytochrome *c* site and thus, at low dye concentrations, the measured rate represents largely the reaction with cytochrome *c*. That this is the case may be readily shown by the following facts: the flatter curve is non-competitively inhibited by amytal or antimycin A (open circles), as expected from the fact that these inhibitors prevent the flux of electrons to cytochrome *c*. It is competitively activated by cyanide and azide (closed circles), because these inhibitors inhibit the flux of electrons via cytochrome oxidase to O_2 and thereby increase the flux of electrons to ferricyanide. The activation is competitive with respect to

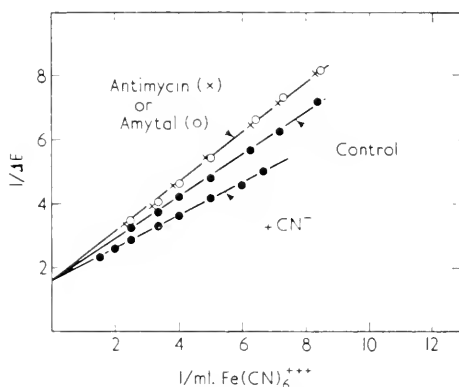


FIG. 2. Ferricyanide assay of DPNH oxidation by ETP in the presence of 2×10^{-4} M DPNH. Conditions as in Fig. 1.

ferricyanide, since at infinite ferricyanide concentration all the electrons would flow to the dye. The steep part of the curve represents the sum of *both* reaction sites of ferricyanide. When the values from the extrapolation of the flat curve are subtracted from the experimental values obtained at high ferricyanide concentrations, the inhibition by antimycin and amytal and the activation by cyanide or azide disappear in accord with the fact that these inhibitors do not affect the DPNH-flavoprotein-ferricyanide reaction sequence.

Attention should be called to the fact that the higher the concentration of ferricyanide employed, the greater the contribution of the dehydrogenase site to the measured activity. Thus it is clearly desirable to employ as high a concentration of the electron acceptor as compatible with the optical arrangement when working at fixed concentrations of the oxidant. In heart particles high concentrations of ferricyanide do not appear to be

inhibitory to DPNH dehydrogenase, although the situation is quite different in liver [15]. Since at high ferricyanide concentrations the curve is very steep, however, for accurate and reliable assays it is clearly desirable to determine the activity at infinite ferricyanide concentration.

The biphasic nature of the curve relating activity to ferricyanide concentrations at or above 6×10^{-4} M DPNH (Fig. 1) is not seen at low (2×10^{-4} M or less) DPNH concentrations (Fig. 2). The break in the curve also disappears on solubilization of the dehydrogenase (Fig. 3), as expected from the fact that this procedure separates the flavoprotein from the respiratory chain and thus leaves only one reaction site for ferricyanide.

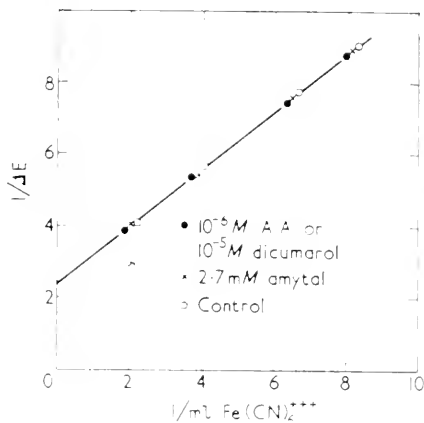


FIG. 3. Ferricyanide assay of dehydrogenase after solubilization with phospholipase A.

One of the many reasons why assays of this enzyme conducted at fixed ferricyanide concentrations tend to be unreliable is the narrow region of DPNH concentrations in which *apparently* optimal activity is observed. As shown in Fig. 4, both the DPNH oxidase and the DPNH-ferricyanide assays are seriously inhibited by substrate concentrations in excess of 1.5 to 2×10^{-4} M. While inhibition of DPNH dehydrogenases and DPNH cytochrome reductases by excess substrate is a fairly common finding, it is interesting to note that the inhibition, at least with the enzyme under discussion, is competitive with respect to the electron acceptor (Fig. 5). Thus the inhibition by moderately high DPNH concentration disappears at infinite concentration of ferricyanide.

Returning for the moment to Fig. 3, it may be noted that the soluble dehydrogenase employed here is completely insensitive to antimycin A and to amytal. It may be remembered that ETP, the starting material employed for the extraction of the enzyme, is 100% inhibited by amytal

and antimycin A in the DPNH oxidase assay and that the reduction of ferricyanide by ETP is also inhibited by these reagents as far as the cytochrome *c* site is concerned (Fig. 1). That the soluble flavoprotein is antimycin-insensitive is not surprising, since this inhibitor is thought to

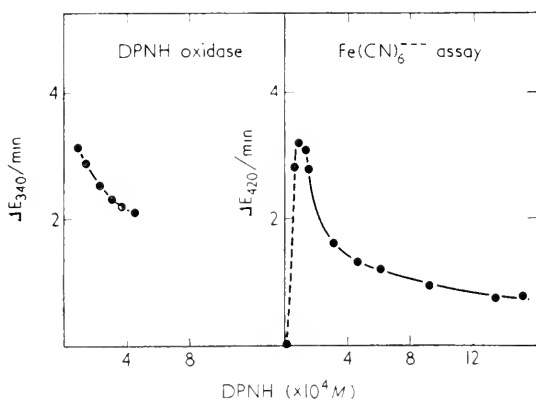


FIG. 4. Inhibition of DPNH oxidase and of DPNH dehydrogenase activities by excess substrate. *Left*: DPNH oxidase assay at 30 ; 120 μ moles phosphate, pH 7.4, 0.06 mg. protein (DPNH oxidase, Crane, *et al.* [11]), and DPNH as indicated in 3 ml. volume. *Right*: Ferricyanide assay at fixed acceptor concentration. Same conditions except that 0.09 mg. protein and 2.5 μ moles $\text{K}_3\text{Fe}(\text{CN})_6$ were present in each cuvette. Reaction time in both experiments about 30 sec.

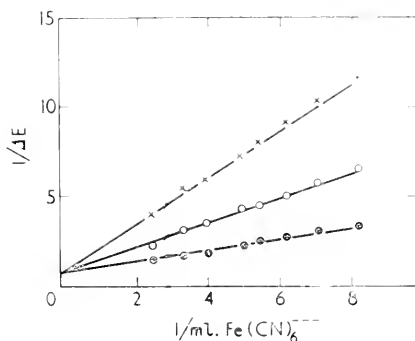


FIG. 5. Ferricyanide assay of soluble DPNH dehydrogenase at various concentrations of DPNH. For assay conditions see Fig. 1. The DPNH concentrations were: 1.5×10^{-4} M, ●—●; 3.0×10^{-4} M, O—O; and 6.0×10^{-4} M, X—X.

act between cytochromes *b* and *c*, but the fact that it is also amytal-insensitive was contrary to expectations, since amytal had been thought to interrupt the flow of electrons from DPNH to flavoprotein [16]. The insensitivity of the isolated dehydrogenase to amytal and the insensitivity

of ETP or DPNH oxidase preparations to this inhibitor in the flavoprotein-ferricyanide interaction (cf. discussion of Fig. 1) suggest that amytal interrupts electron transport between flavoprotein and the respiratory chain, as is also the case in the choline oxidase system of liver [17], and not between DPNH and flavoprotein. Contrary conclusions in the earlier literature were based on the "cross-over technique" which relies on the measurement of the oxidation state of the flavoprotein in the 450-465 $m\mu$ region. As will be documented later in this paper, the application of this technique to DPNH dehydrogenase has some major weaknesses: the difference spectrum is atypical of simple flavoproteins; the extinction coefficient of simple flavoproteins at 465 $m\mu$ is not applicable to this enzyme; and it is not even certain that the flavin is fully reduced in normal catalysis.

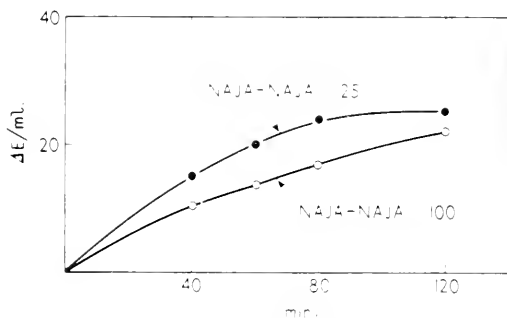


FIG. 6. Progress of solubilization of DPNH dehydrogenase by phospholipase A. Aliquots of a DPNH oxidase preparation [11] were incubated at 30°, pH 7.4, with *Naja naja* venom as a source of phospholipase. The ratios indicated define the mg. weight of venom employed per mg. protein in the particulate preparation (determined by biuret reaction, using a coefficient of 0.095 for 1 mg. protein per 3 ml., 1 cm. light path, 540 $m\mu$). At various times aliquots were rapidly cooled to 0°, centrifuged at 105,000 $\times g$ for 30 min., and the supernatant solution was assayed. Activities are given in arbitrary units on the ordinate.

Turning now to the isolation and characteristics of the dehydrogenase, Fig. 6 shows the progress of solubilization of the enzyme, starting with ETP, at two levels of cobra venom. Compared with brain α -glycerophosphate dehydrogenase [9] and choline dehydrogenase from liver [18], the level of phospholipase A (cobra venom) required for extensive solubilization of DPNH dehydrogenase is quite high and the progress of the reaction under the same conditions is rather slow. These observations are in accord with King and Howard's findings on the conditions necessary for the extraction of the various DPNH oxidizing activities from heart muscle mince [7]. Table I shows the balance of solubilization. It may be noted that the assay is reliable for both particles and the soluble enzyme, since the recovery is satisfactory. By repeating the incubation with a second

batch of venom under the conditions of Table I, 90% or more of the activity may be obtained in solution.

TABLE I
BALANCE OF SOLUBILIZATION

Step	Activity ($\mu\text{M DPNH}/\text{min.}/\text{ml.}$)	Per cent
DPNH oxidase	333	—
Same after venom treatment	303	(100)
Soluble enzyme	187	62
Residue	94	32

Conditions: 80 min. incubation with 1 mg. *Naja naja* per 25 mg. protein at 30°. Solubilization varied from 62 to 78%. Second incubation yields 22% more enzyme in solution.

The fact that the enzyme is in true solution has been shown by the usual criteria: it does not sediment in 1 hr. at $144\,000 \times g$ even after 12 hr. dialysis or repeated freezing and thawing and it may be readily fractionated with $(\text{NH}_4)_2\text{SO}_4$ in a manner characteristic of soluble proteins.

The enzyme has been purified by two cycles of $(\text{NH}_4)_2\text{SO}_4$ fractionation at pH 8.0 and the resulting preparation has a specific activity of about 200 μmoles of DPNH oxidized/min./mg. protein (biuret basis, coefficient = 0.095) at 30°, pH 7.4. Fractionation on calcium phosphate gel or hydroxylapatite has failed to increase the purity further. The enzyme is not held on carboxymethylcellulose at pH 6.8 and it is excluded on Sephadex G75. Fractionation on DEAE cellulose is not feasible, since the enzyme is extremely strongly adsorbed on this ion exchanger. The turnover number per mole of flavin is at least 350 000 at 30°, pH 7.4 in the ferricyanide assay.

Present knowledge of the properties of the enzyme may be summed up as follows. The enzyme is gratifyingly stable and may be preserved for prolonged periods in the frozen state with little or no loss of activity. Even after 96 hr. at room temperature (21°) at the pH of optimum stability (pH 7.5) 80% of the activity remained.

The dehydrogenase does not act on TPNH, nor is TPNH an inhibitor. DPN, however, is a powerful competitive inhibitor; the competition is again with respect to the electron acceptor (Fig. 7). This inhibitory effect of the oxidation product is another reason why accurate assays must be based on the measurement of initial rates at infinite ferricyanide concentration.

The apparent K_M for DPNH, based on assays in which the DPNH concentration was varied at infinite ferricyanide concentration, is $1 \times 10^{-4} \text{M}$

at 30° , pH 7.4 (Fig. 8). It should be noted that this DPNH concentration is only about one-half of that which gives *apparent* optimal activity (Fig. 5) in the ferricyanide assay. While an increase in the initial DPNH concentra-

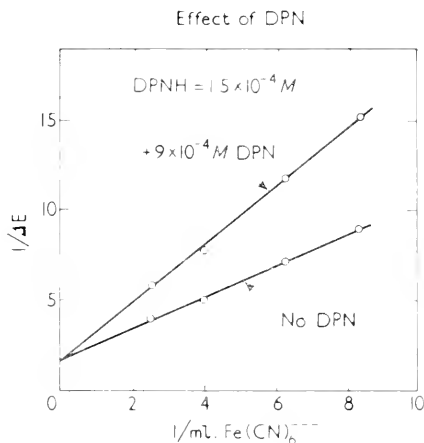


FIG. 7. Competitive inhibition of soluble, purified dehydrogenase by 9×10^{-4} M DPN. Standard ferricyanide assay; DPNH concentration = 1.5×10^{-4} M.

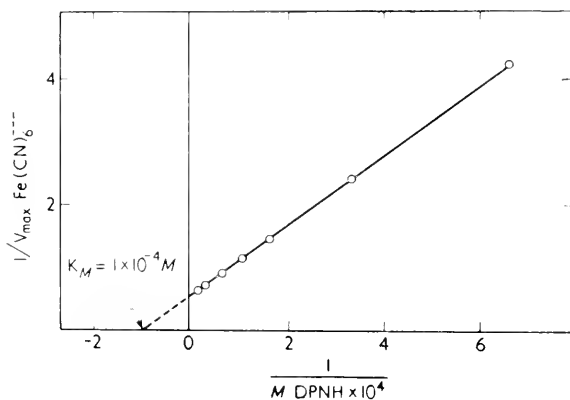


FIG. 8. Lineweaver-Burk plot of effect of DPNH concentration on activity. The values on the ordinate are maximal velocities of ferricyanide reduction (V_{\max} with respect to ferricyanide) corresponding to each concentration of DPNH at pH 7.4, 30° .

tion beyond about 2×10^{-4} M fails to increase the measured rate, even under conditions where the inhibitory effect of excess substrate is eliminated, it is doubtful if this value represents a true "saturation" of the dehydrogenase.

The determination of the "pH optimum" of this enzyme is a particularly difficult task. The ferricyanide assay, as described, functions very satisfactorily in the pH range of about 5.5 to 7.8. In this range double reciprocal plots of activity versus ferricyanide concentration show a definite,

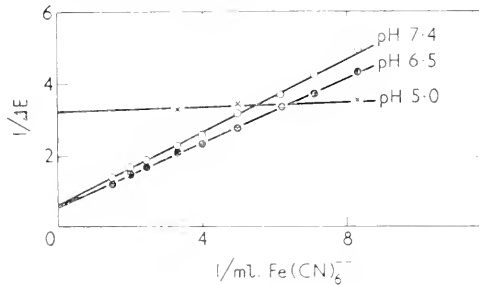


FIG. 9. Ferricyanide assay of soluble, purified enzyme at different pH values.

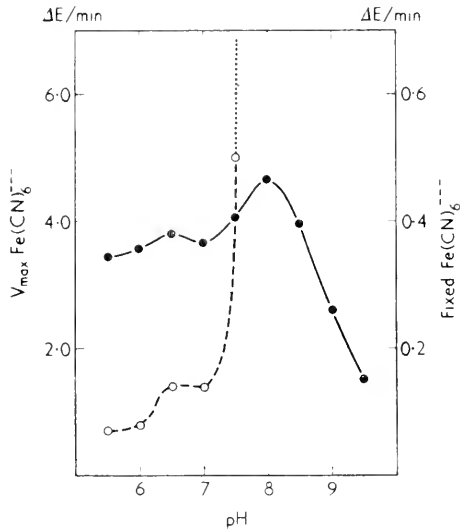


FIG. 10. Effect of pH on the activity of purified DPNH dehydrogenase. Solid line, at fixed (1.66×10^{-3} M) ferricyanide concentration; dashed line, V_{\max} values. The pH values given are those of the reaction mixture at 30°. Buffers: 0.04 M phosphate (pH 5.5 to 8.5) or tris (above pH 8.5).

moderate slope and the reaction kinetics are of zero order, while at pH 5.0 the slope is negligible (Fig. 9) and the reaction assumes first order characteristics with respect to the substrate. As the pH is increased above 8.0, the slope approaches infinity and at pH 8.5 to 9 it intersects the abscissa and thus no satisfactory measure of V_{\max} can be obtained. At present no satisfactory explanation of this complex behaviour is evident.

Within the pH range where assays based on I_{\max} values are reliable (5.5 to 7.8) no definite optimum is attained, but at fixed ferricyanide concentrations (1.66×10^{-3} M or lower) the apparent optimum is around pH 8 (Fig. 10).

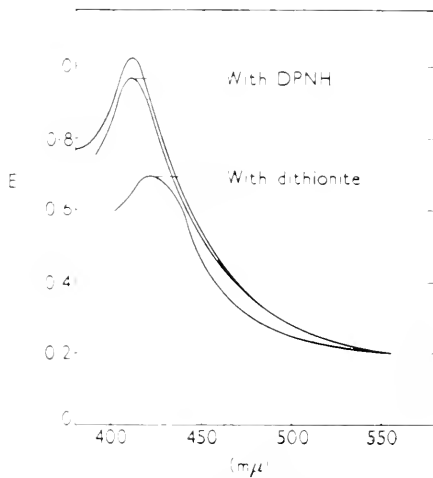


FIG. 11. Absorption spectrum of dehydrogenase in soluble extract, prior to purification and the effects of DPNH and of dithionite on the spectrum. Protein concentration, 3.6 mg. per ml.; pH = 7.15. Recorded with Cary Model 11 spectrophotometer.

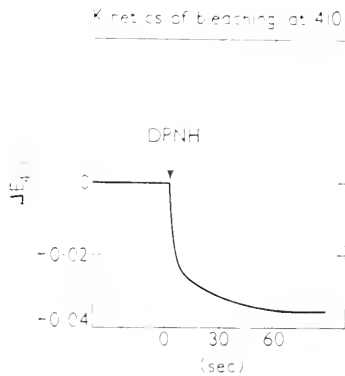


FIG. 12. Kinetics of bleaching by 6.5×10^{-5} M DPNH. Conditions as in Fig. 11.

Certain characteristic features of the absorption spectrum are readily recognizable in the initial soluble extract, prior to purification (Fig. 11). The main peak (upper curve, oxidized enzyme), which at pH 7.4 is located at 410 mμ, is not a Soret band but is strongly reminiscent of that

seen in α -glycerophosphate dehydrogenase [19] and the succinic dehydrogenase of *M. lactilyticus* [20], both of them iron-containing proteins. DPNH and hydrosulphite partially bleach the colour. As compared with succinic dehydrogenase [21], decolourization by the substrate is quite

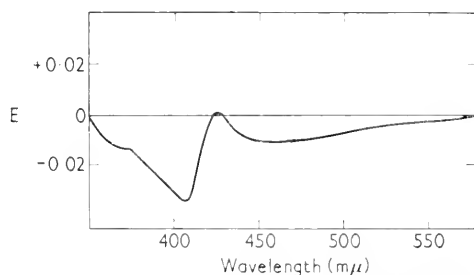


FIG. 13. Difference spectrum (oxidized minus DPNH-treated), replotted from a tracing obtained with Cary recording spectrophotometer. Negative values denote bleaching. Conditions as in Figs. 11 and 12; soluble extract.

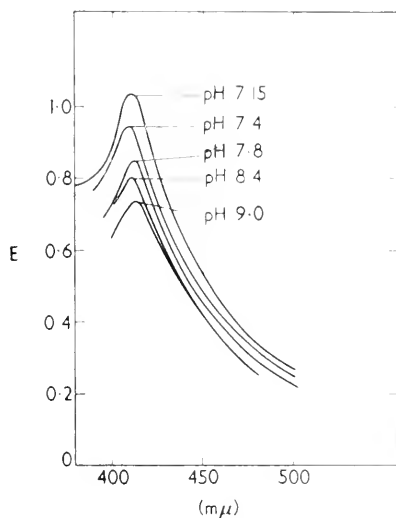


FIG. 14. Shift of absorption spectrum with pH.

rapid (Fig. 12). The difference spectrum resulting from bleaching by the substrate shows minima in the 410 mμ as well as in the flavin region in the initial extract (Fig. 13). In highly purified preparations a single broad minimum centring around 425 mμ is observed and the bleaching is more extensive than could be ascribed to the flavin content.

Before leaving the subject it may be worth mentioning that the absolute position and the height of the peak in the near visible region are strongly dependent on pH (Fig. 14).

The partly purified enzyme (specific activity = 130) was found to be relatively insensitive to inhibition by *p*-chloromercuribenzoate, completely insensitive to dicoumarol (Fig. 3); it did not catalyze the reduction of coenzyme Q₁₀ significantly (with or without added mitochondrial lipid and Triton X-100), and, under the assay conditions recommended by Wosilait [22], the rate of reduction of menadione at I_{\max} was less than 1% of the rate of reduction of ferricyanide. These observations clearly dis-

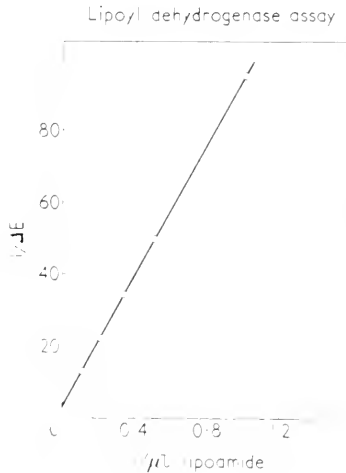


FIG. 15. Lipoyl dehydrogenase assay of DPNH dehydrogenase. Conditions were as recommended by Massey [6]. The abscissa denotes the reciprocal volumes (in μ l.) of 0.058 M lipoamide present in 1 ml. reaction mixture.

tinguish the enzyme from DT diaphorase [23, 24] and from menadione reductase [22].

Under the conditions of the lipoyl dehydrogenase assay employed by Massey [6] the soluble extract obtained on treatment of ETP with phospholipase A shows only a trace of activity on lipoamide (Fig. 15); ratio of activities on ferricyanide and lipoamide, respectively, differ by a factor of about 500 between this preparation and diaphorase (Table II). This trace of lipoyl dehydrogenase activity may well be due to a slight contamination with diaphorase which would be probably removed in the purification procedure. The dehydrogenase may be readily distinguished from DPNH cytochrome *c* reductase [2] by its much greater stability and by the extremely low rate of cytochrome *c* reduction even when assayed under optimal conditions for Mahler's enzyme (Table III). That the residual

TABLE II

COMPARISON OF DPNH DEHYDROGENASE AND DIAPHORASE

Reaction	DPNH Dehydrogenase		Diaphorase
	Rate (μM DPNH/min. ml.)	Ratio $\frac{I'_{\text{max}} \text{Fe (CN)}_6}{I'_{\text{max}} \text{Lipoamide}}$	Ratio $\frac{I'_{\text{max}} \text{Fe (CN)}_6}{I'_{\text{max}} \text{Lipoamide}}$
DPNH + Fe (CN) ₆ ⁺⁺⁺	150	56	—
DPNH + Lipoamide	2.7	—	0.1

Conditions of assay: as per Massey (pH 6.5) [6]. K_m for lipoamide = 2 mM for DPNH dehydrogenase, 5 mM for diaphorase.

activity with cytochrome *c* may represent a trace contamination with Mahler's reductase, rather than a property of DPNH dehydrogenase, is suggested by the fact that the reactivity with cytochrome *c* was inhibited by the same substances at the same concentrations as reported for DPNH cytochrome reductase [25, 26] (Table IV). Such trace contamination would not be surprising in view of the fact that these experiments were carried out with the initial soluble extract prior to fractionation.

TABLE III

CYTOCHROME *c* REDUCTASE ACTIVITY OF PARTIALLY PURIFIED DPNH DEHYDROGENASE

Assay	Activity	Ratio
	(μM DPNH min. ml.) (I'_{max})	$\frac{I'_{\text{max}} \text{Fe (CN)}_6}{I'_{\text{max}} \text{Cyt. } c}$
DPNH + Fe (CN) ₆ ⁺⁺⁺	750	—
DPNH + Cyt. <i>c</i>	0.53	1410

Conditions of assay: as per Mahler and Elowe [25].

Comparison of the properties of the enzyme described with those of the preparation of King and Howard [7] would be interesting but is rendered difficult by the fact that their detailed data, particularly the assay conditions employed, have not yet been published. In view of the similarities in the extraction procedure, it seems very likely that the enzyme here described is one of the DPNH oxidizing activities detected by these workers on chromatographing their extracts on DEAE cellulose [27]. The presence of several components capable of oxidizing DPNH in their preparation is not surprising in view of the heterogeneous constitution of the starting material employed (Keilin-Hartree preparation), which, in turn, might make the definite identification of the individual

TABLE IV

EFFECT OF KNOWN INHIBITORS OF DPNH-CYTOCHROME REDUCTASE ON CYTOCHROME *c* REDUCTION BY DPNH DEHYDROGENASE

Inhibitor	Inhibition (%)
PO ₄ , 0.01 M	78
Pyrophosphate, 0.01 M	85
Ca ⁺⁺ , 0.01 M	78
Mg ⁺⁺ , 0.01 M	76

Conditions: as per Mahler and Elowe [25]; assay at pH 8.5.

flavoproteins after solubilization rather difficult. In the present work this difficulty was circumvented by the expedient of using a starting material known to contain only one DPNH oxidizing activity, the flavoprotein attached to the respiratory chain.

Assuming that the assay conditions are not too dissimilar, the best preparation hitherto obtained in the Detroit laboratory is some eighty to ninety times more active than the purified enzyme described by King and Howard [7]. The major differences between their preparation and ours are that while theirs is very unstable, ours is quite stable; their preparation precipitates at a considerably higher range of (NH₄)₂SO₄ concentrations than does ours [7]; and, finally, that while our enzyme is so thoroughly adsorbed on DEAE cellulose as to render fractionation on this exchanger quite unfeasible, theirs is readily eluted from DEAE cellulose [28]. These differences seem too great to be readily accounted for by the different degrees of purification of the two preparations and, hence, at this time it is uncertain whether the two laboratories are indeed investigating the same enzyme.

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Discussion

ERNSTER: Does your enzyme react with quinones as electron acceptors?

SINGER: We haven't had a chance to test it yet.

ERNSTER: I should like to recall an interesting observation which Dr. Conover and I made some time ago on a non-purified preparation of DPNH oxidase; we found that with vitamin K₃ as terminal electron acceptor we obtained an appreciable amytal-sensitivity, whereas with 1,4-naphthoquinone the amytal-sensitivity was only marginal (cf. Ernster, this volume, Table 7, page 150).

Note added in proof. The homogeneous enzyme contains about 16 atoms of non-haem iron and 1 mole FAD per 10⁶ g. protein. The pH optimum range, as determined by a transhydrogenase assay, is pH 8 to 9. In this range the turnover number per mole of flavin is 1.3 million per minute at 30°.

Reversal of Electron Transfer in the Respiratory Chain*

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1. Energy-linked DPNH reduction

GENERAL FEATURES OF THE REACTION

It was observed some time ago in collaboration with Dr. G. R. Williams [1] that addition of succinate to mitochondria oxidizing a DPNH-linked substrate caused a significant increase of pyridine-nucleotide reduction. This phenomenon was especially clear in guinea-pig-kidney and rat-heart mitochondria studied with Dr. G. R. Hollunger [2, 3] and was most recently

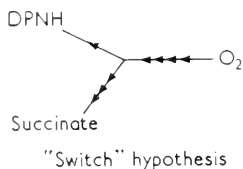


FIG. 1. Diagram illustrating how competition between DPNH and succinate for oxidizing equivalents from cytochrome chain could lead to increased pyridine-nucleotide reduction; the number of arrowheads indicates the proportion of electron transfer which might flow in the chain and its two branches. (MD 102).

observed in pigeon-heart mitochondria with Dr. B. Hagihara [4]. The result has been confirmed by chemical assays in a number of laboratories [5-10].

Such a phenomenon might readily have been ascribed to a competition between succinate and DPNH for oxidizing equivalents in the cytochrome portion of the chain (Fig. 1).† On this basis one would have expected that

* This research was supported in part by the National Science Foundation.

† The "switch" hypothesis is discussed in some detail by Birt and Bartley [21] although kinetic studies were not possible with their analytical methods. This hypothesis received only a short discussion previously [3] where the general case of a "simple kinetic explanation" based upon a greater speed of pyridine-nucleotide reduction by succinate appeared to be inadequate. Here we elaborate our views and present additional evidence.

succinate would deprive DPNH of the oxidizing capacity of the chain and lead to a greater degree of pyridine-nucleotide reduction. However, many features of the reaction called our attention to a need for closer study. For example, the rate at which succinate produced increased reduction of pyridine nucleotide appeared to be slow compared with that at which

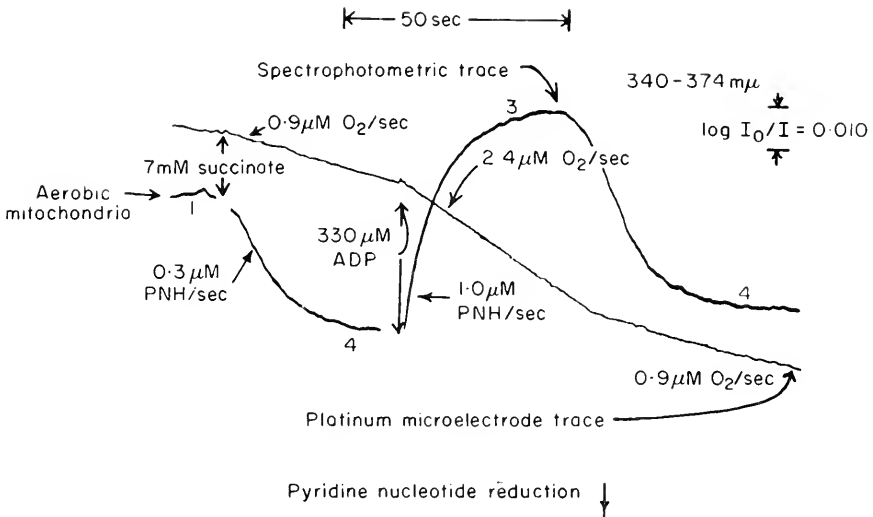


FIG. 2. Illustrating increased reduction of pyridine nucleotide in a suspension of rat-liver mitochondria caused by addition of 7 mM succinate. Absorbancy changes measured spectrophotometrically by double-beam spectrophotometer and respiration by vibrating platinum microelectrode. Downward deflection upon addition of reagent indicates increased light absorption at 340 $m\mu$ relative to 374 $m\mu$. Diagram indicates final concentrations of reagents added, respiratory rate in μ moles/l./sec. and increment of oxygen taken up during phosphorylation of 330 μM ADP. Rates of pyridine-nucleotide reduction also given in μ moles/l./sec. The metabolic states of mitochondria are indicated by numerals 1-3-4. Rat-liver mitochondria diluted in isotonic salt medium to concentration of approximately 2 mg. protein/ml. at pH 7.4, temperature 25° (Expt. 332-2).

succinate could intercept oxidizing equivalents from the respiratory chain and was no more rapid than the rate at which pyridine nucleotide could be reduced by DPNH-linked substrates. Another puzzling feature of the reaction was that it was slowed by addition of very low concentrations of uncoupling agents and completely inhibited by larger concentrations [3]. This too seemed inconsistent with a simple competitive reaction which should also occur at higher respiratory rates caused by addition of the uncoupling agents. Further doubts as to a simple explanation of the reaction

were afforded by the inhibitory effects of amytal upon the rate of pyridine-nucleotide reduction caused by succinate. Since amytal inhibits DPNH oxidation by the mitochondrial respiratory chain [11, 11a], one would have expected that the effectiveness of succinate in intercepting oxidizing equivalents would have been even greater in the presence of this inhibitor. On the other hand, the observation of Klingenberg *et al.* [5] that α -glycerophosphate causes enhanced pyridine-nucleotide reduction in locust flight-muscle mitochondria was not inconsistent with the hypothesis of a simple competitive reaction.

Our doubts about the simplicity of this mechanism led us to carry out an extensive study of the nature of succinate-linked pyridine-nucleotide reduction in a variety of mitochondrial preparations under diverse conditions with emphasis on the kinetics of intramitochondrial reactions.

An example of the pyridine-nucleotide reduction by succinate in rat-liver mitochondria was presented in 1956 [1] and illustrated the important features of the reaction (Fig. 2). These mitochondria containing endogenous substrate show, upon addition of succinate, increased pyridine-nucleotide reduction indicated by the trace's downward deflection corresponding to increased absorption at $340\text{ m}\mu$ measured with respect to $374\text{ m}\mu$. The reduction rate is not rapid and a steady state is obtained in about 1 min. The increase of respiration is not great, the initial rate of $0.56\ \mu\text{M O}_2/\text{sec.}$ rising to $0.9\ \mu\text{M O}_2/\text{sec.}$ Characteristic of the reduction reaction is its reversal by ADP, illustrated here by the trace's abrupt upward deflection upon addition of that reactant and increased pyridine-nucleotide reduction upon exhaustion of the added ADP. In view of the possible obscuration of DPNH reduction by the concomitant reduction of TPNH in liver mitochondria [5, 12, 13] we have been studying heart and kidney preparations in preference to those of liver since 1956 because of their low TPNH content [13]. The percentage increase of DPN reduction obtained on adding succinate to guinea-pig-kidney mitochondria runs as high as fourfold, making such material ideal for kinetic and stoichiometric studies [2].

Figure 3 gives an example of succinate-linked pyridine-nucleotide reduction in guinea-pig-kidney mitochondria from work with Dr. Hollunger [2]. Mitochondria are pretreated with 4 mM glutamate and about 25% of the total DPN is reduced in state 4. At this point addition of succinate causes a striking increase in pyridine-nucleotide reduction, as indicated by the large downward deflection of the trace. There appears to be a transient respiratory acceleration upon succinate addition to the glutamate-treated mitochondria. Thereafter net respiratory acceleration on succinate addition to the glutamate-treated material is not extremely large. Note that the rate at which DPN is reduced is comparable (on a 2-electron basis) to the State 4 respiration rate in the presence of succinate

(0.28 compared to 0.30). As in the case of liver mitochondria, reduced pyridine nucleotide shows a typical cycle of oxidation and reduction upon addition of ADP. But the initial rate of DPNH oxidation is small compared with the steady-state rate of oxygen utilization (0.56 compared with 2.0 in 2-electron equivalents). Thus the significant features of this reaction are the relatively slow changes in pyridine-nucleotide reduction states, which lead nevertheless to very large magnitudes of changes in steady state.

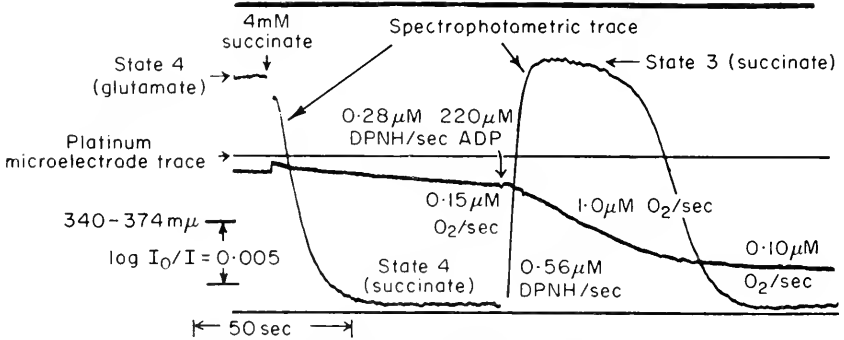


FIG. 3. Illustrating increase of pyridine-nucleotide reduction caused by adding succinate to glutamate-treated guinea-pig-kidney mitochondria. Downward deflection of trace indicates increased absorbancy at 340 m μ measured with respect to 374 m μ . Rates of pyridine-nucleotide reduction and oxygen utilization indicated in μ moles/l./sec. Mitochondria diluted in sucrose-phosphate medium to concentration of 0.6 mg. protein/ml., pH 7.4, temperature 25°. (Expt. 683-1). (Reproduced with permission of *The Journal of Biological Chemistry*.)

While the above experiments showed the important role of succinate in activating DPN reduction in mitochondria they did not clearly rule out the possibility that addition of succinate increased the concentration of a DPN-linked substrate, for example malate, according to the sequence of the citric acid cycle (Fig. 4). This hypothesis is largely ruled out in



FIG. 4.

experiments illustrating the abrupt inhibitory effect of malonate upon succinate-linked DPN reduction. For instance in experiments such as those recorded in Figs. 2 and 3 addition of malonate causes an abrupt decrease of pyridine-nucleotide reduction to the level previously obtained in the presence of the DPN-linked substrate only. That this inhibition occurs with no measurable induction period, as would have been expected for a mechanism which depended upon accumulation of DPN-linked substrate, rules against the simple hypothesis outlined in Fig. 4.

Experiments of the type indicated by Figs. 2 and 3 suggest an essential role for succinate in particular, and for a flavin-linked substrate in general, in reduction of a considerable portion of mitochondrial pyridine nucleotide. However, such experiments do not plainly separate the electron-transfer and energy requirements for the reaction.

An energy requirement for this reaction is apparent from two stand-points. First, there is at least a 300-mv. unfavourable potential difference between the succinate-fumarate and the DPN-DPNH couples and second, the sensitivity of the reaction to uncoupling agents suggests that the energy requirement was met by internal high-energy intermediates of oxidative phosphorylation (Fig. 5). In more recent experiments at the Johnson Foundation, preparations of pigeon heart mitochondria [14] have been studied in which succinate causes no appreciable pyridine-nucleotide reduction in the absence of ATP and, more important, ATP causes no appreciable pyridine-nucleotide reduction in the absence of succinate.



FIG. 5.

Figures 6 and 7 illustrate the properties of succinate-linked pyridine-nucleotide reduction in pigeon-heart mitochondria where the electron-donor and energy-donor requirements are separable. In Fig. 6 pigeon-heart mitochondria are suspended in an aerobic medium containing 0.27 M mannitol, 0.03 M sucrose, 0.02 M "tris" buffer, pH 7.4, free of added magnesium and phosphate.* Under these conditions succinate addition causes no downward deflection of the spectrophotometric trace; there is no measurable pyridine-nucleotide reduction. However, upon addition of 100 μM ATP there is an abrupt downward deflection of the trace, indicating reduction of DPN. This reaction continues for about 2 min. In a similar experiment under the same conditions mitochondria are pretreated with 78 μM ATP (Fig. 7). There is only a very small downward deflection of the trace amounting to about 4% of the total pyridine nucleotide. However, upon addition of succinate there is an initial rapid reduction of DPN which then proceeds at a slower rate.

These experimental results can now be considered against the background of the various hypotheses that have been considered. It is apparent that the "switch" hypothesis (Fig. 1) is inapplicable to these experimental conditions since succinate alone causes no measurable pyridine-nucleotide reduction.

An hypothesis suggesting that only succinate is required for pyridine-nucleotide reduction is apparently inconsistent with these data, as is one

* Thanks are due to Mr. K. Kaminker and Miss H. Diefenbach for pigeon-heart preparations and to Dr. U. Fugmann for "digitonin" preparations.

suggesting that only ATP is required for pyridine-nucleotide reduction. These considerations eliminate hypotheses which postulate that DPN-linked substrates are acting directly or through an ATP-activated step, since succinate or ATP alone should cause the observed effects.

A remarkable feature of the reaction and one which seems to differ considerably from the results so far obtained by Klingenberg and Azzone (this meeting) is the low concentration of ATP required for pyridine-nucleotide reduction. Under favourable conditions it has been observed

Fluorescence excitation 365m μ
Fluorescence measurement 450m μ

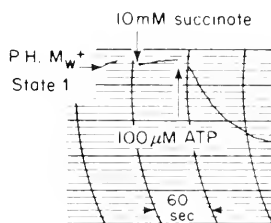


FIG. 6.

FIG. 6. Illustrating separation of electron and energy-transfer requirements for succinate-linked pyridine-nucleotide reduction. Addition of succinate alone causes no fluorescence change, while 100 μ M ATP causes a large fluorescence increase corresponding to pyridine-nucleotide reduction. Pigeon-heart mitochondria suspended in sucrose-"tris"-mannitol medium, pH 7.4, temperature 26° (Expt. 133d). (Reproduced with permission of *The Journal of Biological Chemistry*.)

Fluorescence excitation 365m μ
Fluorescence measurement 450m μ

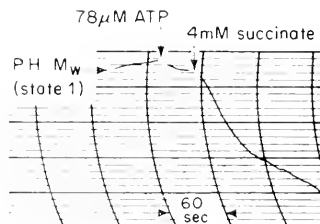


FIG. 7.

FIG. 7. Illustrating separate energy and electron-transfer requirements for succinate-linked pyridine-nucleotide reduction. Pigeon-heart mitochondria suspended in mannitol-sucrose-"tris" medium, pH 7.4, temperature 25°. Downward deflection of fluorescence trace indicates increase of pyridine-nucleotide reduction (Expt. 133d). (Reproduced with permission of *The Journal of Biological Chemistry*.)

that as little as two ATPs per DPNH are required [15]. This result is obtained under relatively poor conditions for optimal efficiency of the reaction since the mitochondria are capable of breaking down ATP without expending its energy in the reduction of pyridine nucleotide. The small ATP requirement for DPN reduction also sets a value for any other substances that might react with ATP. First, the conditions of the experiment are such that about 20 μ M DPN is reduced on addition of about 60 μ M ATP. Thus readily detectable chemical changes are caused by the ATP reaction which can easily be confirmed by extraction of the mitochondria and subsequent analysis.

PATHWAY OF SUCCINATE-LINKED DPN REDUCTION

In view of the evidence in favour of requirements for electron and energy donors in this reaction, it is important to consider the pathway by which electron and energy transfer might occur. With regard to electron transfer, the central question is the nature of the actual electron donor to DPN. Two hypotheses may be considered (Figs. 8, 9). In Fig. 8 the pathway of electron transfer from succinate to DPN involves carriers of the

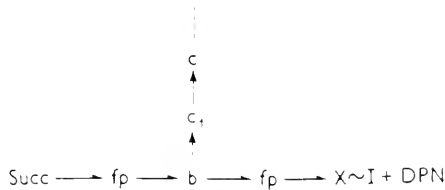


FIG. 8.

respiratory chain. In fact, electrons are depicted to travel part of the way toward the oxygen and then to be bypassed into an energy-requiring pathway involving pyridine-nucleotide reduction. In this portion of the pathway, electron transfer under the influence of ATP would proceed in the reverse of the usual direction in oxidative reactions. Such a pathway would be expected to show inhibitor sensitivity typical of this portion of the electron pathway—a sensitivity to amytal and possibly to antimycin-A. The enzyme system involved in this reaction would presumably be tightly bound to the mitochondrial structure.

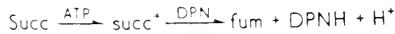


FIG. 9.

Figure 9 shows the second hypothesis by which ATP activates succinate or some immediate oxidation product to an energetic form with suitable thermodynamic properties for direct reduction of DPN by a typical dehydrogenase reaction. This hypothesis differs from that of Fig. 4 only in that a novel reaction product of succinate is postulated which has so far not been identified. This reaction mechanism would be expected to be insensitive to inhibitors of electron transfer through the respiratory chain, such as amytal and antimycin-A, and presumably would be isolable in soluble form.

Figure 10 illustrates that the cycle of oxidation and reduction of succinate-linked pyridine nucleotide shown in Fig. 3 is greatly affected by pretreatment with 0.8 mM amytal. In Fig. 3 we see that oxidation proceeds immediately upon addition of ADP and reduction occurs coincident

with the diminution of respiratory rate following phosphorylation of ADP. In Fig. 10, however, the amytal-treated material shows pyridine-nucleotide oxidation that does not reach a steady state for approximately 1 min. In fact, most of the ADP has been phosphorylated before DPNH oxidation is completed. Subsequently, upon exhaustion of ADP, pyridine-nucleotide reduction proceeds for approximately 1 min. While it would be expected that oxidation of succinate-linked DPNH would be sensitive to amytal, in view of the general sensitivity of DPNH-linked oxidations to this inhibitor, it is surprising that the reduction is so severely inhibited unless the reaction is proceeding by reversed electron transfer through the same carrier as that through which the oxidation reaction is occurring.

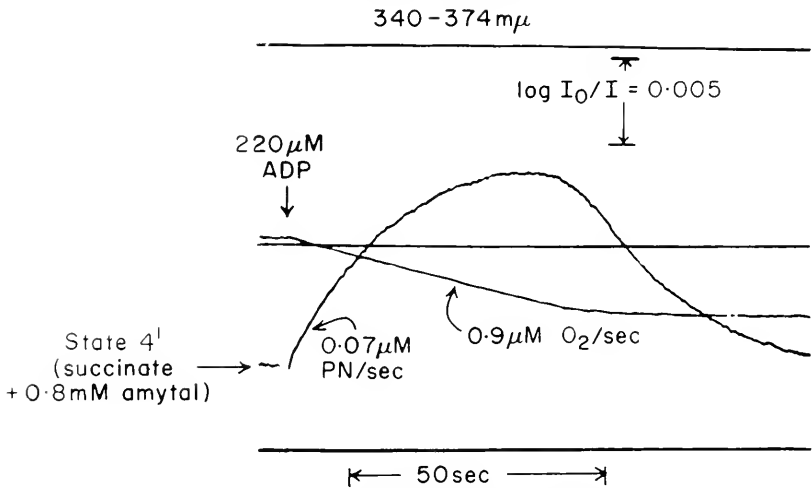


FIG. 10. Effect of amytal upon cycle of oxidation and reduction of succinate-linked reduced pyridine nucleotide in presence of 0.8 mM amytal. Experimental conditions identical to those in Fig. 3 and comparison of rates of reaction in both cases is possible (Expt. 683-3). (Reproduced with permission of *The Journal of Biological Chemistry*.)

We interpret this experiment as identifying an amytal-sensitive reaction in not only the oxidation but also the reduction of pyridine nucleotide. This result strongly supports the idea that energy-linked reversal of electron transfer through the respiratory carriers is involved in the succinate-linked reductions of DPN (Figs. 5, 8).

The question of the level to which electron transfer proceeds toward oxygen before it is bypassed into pyridine-nucleotide reduction is suggested by experiments in which reduction of DPN by succinate and ATP is highly inhibited by hydroxyquinoline-N-oxide (HOQNO) or antimycin-A. At the present time experimental data suggest that electron transfer proceeds to the antimycin-A-sensitive point (Fig. 8).

Also of considerable interest is the pathway for energy transfer from ATP; according to the mechanism of Fig. 9 this would *not* necessarily be through those transfer reactions involved in oxidative phosphorylation and currently associated with ATPase and ATP-³²P exchange reactions. Figure 11 illustrates the pathway of energy transfer following reversal of oxidative phosphorylation. It is apparent that such a sequence of reactions would be sensitive to accumulation of reaction products such as ADP. This has been demonstrated. As mentioned above, uncoupling agents are potent inhibitors of succinate-linked pyridine-nucleotide reduction since they hydrolyze the high-energy intermediates X~I. Magnesium also inhibits, presumably by activating the breakdown of one of the energy-rich intermediates. Further experiments also show that the reaction is largely inhibited by low concentrations of oligomycin. On the basis of such data, it is apparent that the pathway of energy transfer is essentially a reversal of oxidative phosphorylation. This consideration casts further

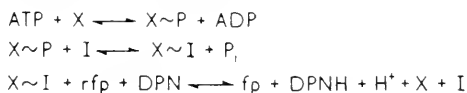


FIG. 11.

doubt upon the feasibility of the mechanism of Fig. 9, which implies that activation of succinate might follow a pathway other than that employed in oxidative phosphorylation.

In summary we can put forth many experimental data indicating that the pathways of electron and energy transfer in succinate-linked pyridine-nucleotide reduction are similar or identical to those of oxidative phosphorylation, the only difference being that a reversal of the process of oxidative phosphorylation, in both the energy and electron-transfer steps, has been revealed by these experiments.

A schematic diagram of the assembly of electron and energy-transfer reactions by which this reaction may be possible is indicated in Fig. 12. The important feature of this mechanism is that it does not require that all DPNH reduction and oxidation proceed through the succinate-linked pathway, but allows this to be a side pathway which may involve a small fraction of the total electron transfer, as is consistent with available kinetic data. In addition, an attempt has been made to indicate the possible participation of quinone in electron transfer between flavin and cytochrome *b* in the forward or reverse directions. Since the function of quinone has not been conclusively proved in either one of these pathways, the mechanism is arranged so that by-passes around the quinone are feasible. A third feature of this scheme is a mechanism by which a larger amount of pyridine nucleotide can be reduced in the energy-linked pathway than in the usual dehydrogenase-linked pathway of DPN reduction.

The succinate-linked mitochondrial pyridine nucleotide is postulated to be separated from the remainder of the DPN by a compartment—possibly the cristae and matrices of the mitochondria are involved. This compartmentation implies that electrons donated by a DPN-linked substrate cannot readily enter the succinate-linked pyridine-nucleotide pool while those from succinate can.

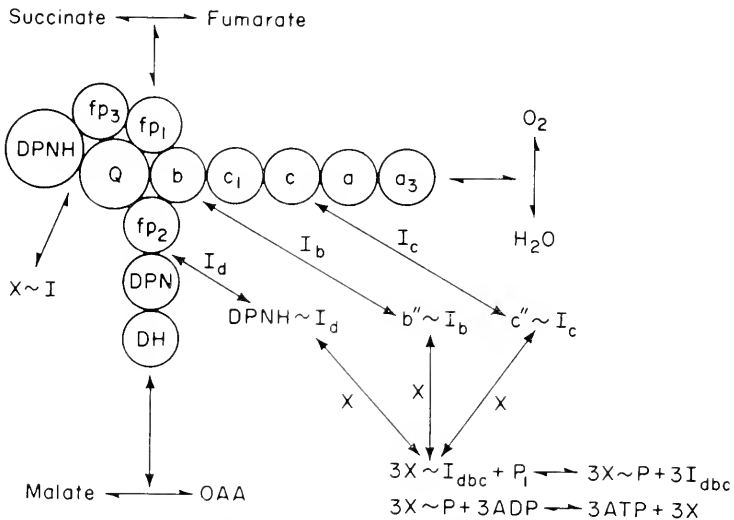


FIG. 12. Schematic diagram of electron-transfer pathways in respiratory chain involving succinate-linked pyridine-nucleotide reduction. This diagram is similar to those presented earlier [1, 18, 19] and includes the quinone component [20]. (Reproduced with permission of the *Journal of Biological Chemistry*.)

2. Energy-linked cytochrome oxidation

Since the preceding considerations demonstrate reverse electron transfer in a branch of the respiratory chain, we have actively considered the possibility that flavoprotein may be oxidized in DPN reduction as indicated in Fig. 11, provided experimental conditions could be arranged so that pyridine nucleotide was oxidized and flavoprotein reduced. A suitable condition for this can be obtained by antimycin-A or quinoline oxide inhibition of the respiratory chain, reinforced by hydrosulphide inhibition of the oxidase. The plan for such an experiment is indicated by Fig. 8. If electrons have already been transferred up to the level of cytochrome *b* and flavoprotein, so that the flavoprotein involved in DPN reduction is already reduced, then indeed addition of ATP should be all that is needed to cause pyridine-nucleotide reduction with a concomitant oxidation of flavoprotein. It has been observed in pigeon-heart mitochondria that treatment of the aerobic suspension with 4 mM succinate and sufficient

hydrosulphide or cyanide to block respiration causes no measurable pyridine-nucleotide reduction, as observed fluorometrically with 365 m μ excitation and 450 m μ measurement [16]. Under such conditions reduction of flavin, quinone and cytochrome components can be observed spectrophotometrically. Thus the respiratory chain is in a condition where the effect of ATP on the sequence of reactions depicted by Fig. 8 could be observed readily. Figure 13 shows that flavoprotein is oxidized at the

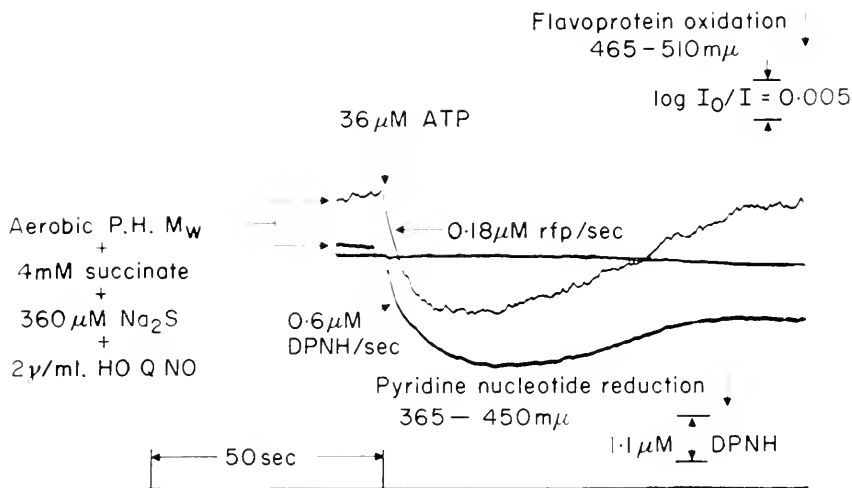


FIG. 13. Flavoprotein oxidation and pyridine-nucleotide reduction caused by ATP addition to pigeon-heart mitochondria inhibited with hydroxyquinoline-N-oxide and sodium sulphide. Mitochondria suspended in mannitol-sucrose-"tris" medium, pH 7.4, temperature 26°, protein concentration 1.2 mg. ml. (Expt. 173). (Reproduced with permission of *Nature*.)

same time that pyridine nucleotide is reduced upon addition of ATP to the HOQNO- and Na₂S-treated mitochondria. This result affords strong support for the reaction of Figs. 8 and 11. It is also of interest that under these conditions the reaction of ATP with the rfp-DPN couple is so rapid that succinate cannot maintain flavoprotein reduced against the oxidizing effect of added ATP. However, when the DPN has been reduced, the oxidized flavoprotein is reduced by succinate toward its initial level.

An experiment of this type suggests the possibility of generalized reversal of electron transfer between all couples of the respiratory chain involved in oxidative phosphorylation. It is apparent that if reduced flavoprotein can be oxidized by ATP a similar effect should be observed at the level of the cytochromes under appropriate conditions.

We therefore repeated an experiment similar to that of Fig. 13 except

that we observed at wavelengths appropriate to cytochrome c^* and omitted the quinoline oxide. Thus Fig. 14 represents an experiment in which pigeon-heart mitochondria are pretreated with 4 mM succinate and sufficient hydrosulphide to block respiration. Under these conditions cytochrome c is completely reduced in about 1 min. As explained above, pyridine nucleotide is not reduced. Upon addition of 530 μM ATP, pyridine-nucleotide reduction in agreement with Fig. 13 is observed. At the same time we recorded an abrupt upward deflection of the spectrophotometric trace indicating an oxidation of cytochrome c to an extent of 71% of the total. While pyridine-nucleotide reduction proceeds exponentially toward a highly reduced level, the response of cytochrome c to ATP

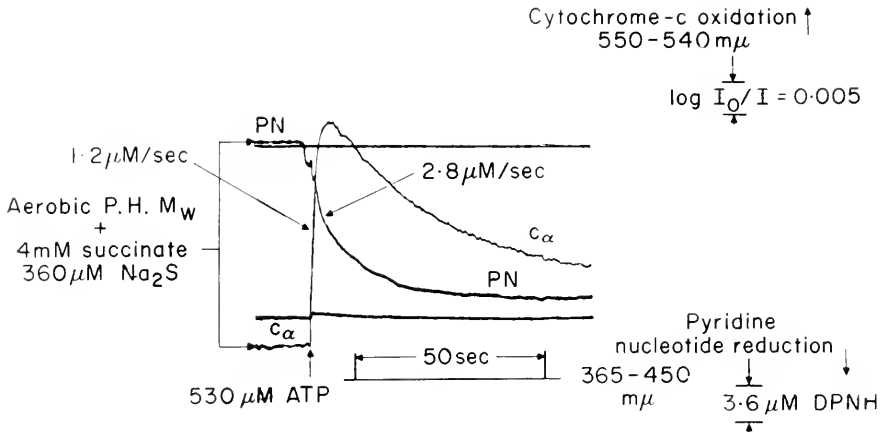


FIG. 14. Cytochrome c oxidation and pyridine-nucleotide reduction in succinate- and sulphide-treated mitochondria (concentrations indicated on figure). Pigeon-heart mitochondria suspended in mannitol-sucrose-"tris" medium, pH 7.4, temperature 26°, protein concentration 1.1 mg/ml. (Expt. 185b-3).

addition is cyclic and reduction toward the initial level proceeds rapidly. Other experiments show cytochrome a to be oxidized as well, cytochrome b showing little initial change and then a slow reduction* which is complete at about the same time as that of pyridine nucleotide. Studies

* Lundegardh [22] has reported an effect of ATP (and fumarate) upon the interaction of cytochromes b and " dh " in anaerobic wheat roots, cytochrome " dh " being "turned over into a state of strong reduction under the influence of ATP (and fumarate) simultaneously with cytochrome b remaining more oxidized". The interpretation of this interesting result is rendered ambiguous by the fact that the existence of cytochrome " dh " in wheat roots and other plant or animal tissues has not been confirmed by other workers [23] nor by us using liquid nitrogen spectroscopy of wheat-root mitochondria (B. Chance and W. Bonner, Jr., unpublished observations).

of quinone under similar experimental conditions are difficult because of the absorbancy change caused by addition of ATP at 275 $m\mu$. However, preliminary studies show quinone to be oxidized and it may be an important component of the couple involved in DPN reduction. Such changes are consistent with the idea that ATP is entering the respiratory chain in the flavoprotein-pyridine nucleotide region as well as the cytochrome region. Similar effects have been demonstrated in the presence of various respiratory inhibitors, for example cyanide, and even in the presence of dithionite. It is found that dithionite does not readily penetrate the mitochondrial membrane and thus mitochondrial pyridine nucleotide is not initially reduced, permitting time for studies similar to those indicated in Fig. 14. Various types of mitochondria show this reaction, for instance rat-liver mitochondria and "digitonin" particles have been tested.

The specificity of the reaction for various nucleotides has been investigated and found to be highly specific for ATP; GTP, UTP, CTP, and ITP show no measurable reduction of pyridine nucleotide or oxidation of cytochrome *c* under conditions similar to those of Fig. 14. These data support those already indicating that ATP is interacting with the respiratory chain through the pathway by which oxidative phosphorylation occurs.

Discussion

As illustrated by Fig. 14 the interaction of ATP with the cytochromes appears to be rapid, but is much slower than the rate of ferrocyclochrome *c* oxidation obtained by the rapid flow apparatus. The reaction is, however, quite sensitive to inhibitors of the pathway illustrated by Fig. 11 and it has been found that ADP, oligomycin, and phosphate inhibit the oxidation of cytochrome *c* as well as the reduction of DPN. Thus the pathway by which ATP enters the respiratory chain is identified with the pathway of oxidative phosphorylation by its inhibitor sensitivity and nucleotide specificity. This pathway, which has been identified with ATPase and ATP- ^{32}P exchange activities, is acting under these conditions to transfer energy from ATP into oxidation-reduction couples of the respiratory chain—an ATP-electron transferase activity. That the activity of this enzyme system can be measured in the intact mitochondria without activating hydrolysis of one of the intermediates in the sequence of Fig. 11 presents tremendous advantages for two kinds of experiments: (1) to determine the maximal activity of the ATP-electron transferase pathway, and (2) to evaluate the effectiveness of reconstituted phosphorylation systems such as those of Polis [17], Pullman, and Lehninger (this symposium).

The efficiency with which ATP can convert its energy into electron transfer is of considerable theoretical and practical interest, particularly in connection with theories of active transport and photosynthesis. We have

therefore titrated the oxidation of cytochrome *c* with ATP and obtained a sigmoid titration curve, presumably due to preferential expenditure of ATP at other couples at low concentrations of ATP. However, the maximum slope of the graph corresponds to roughly 4 ATPs per electron, over twice the observed value of 1.5 ATPs per electron produced in oxidative phosphorylation. Our experimental value is surely not a minimum value since some ATP is lost in the simultaneous oxidation of other carriers in addition to cytochrome *c* and in hydrolysis of intermediates formed from ATP. Thus the efficiency may under appropriate conditions approach the higher values. It is unlikely that the ATP/*e* value for the reversal of electron transfer would reach the experimentally observed value for oxidative phosphorylation in the forward direction since the efficiency of the latter process is probably less than 100%. In fact an estimate of the efficiency of oxidative phosphorylation can be obtained by the ratio of the two values and on the basis of these preliminary data a value of over 50% is obtained.

In addition to considerable interest in the stoichiometric properties of the ATP-electron transfer interaction, the thermodynamic properties are of importance and preliminary titrations of the extent of oxidation of cytochrome *c* in "phosphate-potential buffers" (ATP/ADP.P_i) have been made. We are for the first time able to study the oxidation levels of cytochromes in the presence of ATP under conditions where electron flow through the respiratory chain is sufficiently small to be negligible. Furthermore, the rate of ATP breakdown due to hydrolysis can be so small that the initial concentration of ATP is practically constant during the measurement of cytochrome concentration. Thus the system can be sufficiently near equilibrium to consider the relationship between phosphate potential and cytochrome oxidation. Experiments similar to those of Fig. 14 but in the presence of varying concentrations of ATP, ADP and P_i show that the oxidation of cytochrome *c* is very sensitive to small amounts of phosphate and ADP and a considerable inhibition of the extent of oxidation can be obtained. Actually it is difficult to obtain 50% oxidation of cytochrome *c* under conditions where the ADP and phosphate concentrations are sufficiently high to insure that equilibrium and not stoichiometric factors are determining.

Preliminary estimates suggest that the phosphate potential necessary to cause cytochrome oxidation does not correspond to the complete free-energy change from DPNH to oxygen but instead to a value that would be expected for a single redox couple involved in oxidative phosphorylation. This possibility is supported by titration of the respiratory chain in the absence of added succinate where the [ATP/ADP]/[P_i] ratio is about 10¹ corresponding only to about 15 kcal. Since spectrophotometric observations of pyridine-nucleotide reduction and the oxidation of cytochrome *c*

were made under these experimental conditions, we have no alternative but to conclude that all the components of the respiratory chain itself were not in equilibrium as an electron-transfer system, but the couples of the respiratory chain were interacting individually with the ATP-electron transfer system, presumably because under these particular experimental conditions the latter reaction is much more rapid than that of electron transfer.

Such an experimental condition calls our attention again to the intense inhibition of electron transfer through the chain attributed to hypothetical "I" compounds. It follows from Figs. 11 and 12 that a high concentration of ATP would lead to a concentration of the "I" compounds sufficient to bind the carriers tightly in their inhibited form [5]. Thus the products of the reaction of the ATP-electron transfer activity are concluded to be the inhibited and not the free forms of the carriers, to explain the low values of "phosphate potential" which can cause half-maximal cytochrome oxidation. However, this hypothesis must be considered a tentative one because of our limited experience with this new phenomenon.

At the present time investigations are under way to locate "crossover points" for the ATP-electron transfer activity and experiments such as those of Fig. 13 suggest an interaction site between DPNH and flavin. The response of cytochrome *b* suggests that crossover points may be found on either side of this component.

Summary

These experiments have attempted to elucidate two pathways by which ATP may be used in activating the reversal of electron transfer through components of the respiratory chain. The first pathway investigated is a succinate-activated branch of the respiratory chain which leads to reduction of the majority of mitochondrial pyridine nucleotide, providing that an energy source such as ATP is available. The specificity of flavin-linked substrates such as succinate has been studied as has the pathway of electron transfer through respiratory carriers. Similarly, the pathway of energy transfer from ATP to DPNH has been shown to involve the transfer system employed in oxidative phosphorylation.

Of more general concern is the observation that ATP can cause oxidation of reduced cytochromes in a magnesium-, phosphate-, and ADP-free system, and in a respiratory chain blocked at the oxygen end by a suitable inhibitor or by the lack of oxygen. This reaction may be observed in spite of the presence of a reducing substrate such as succinate. Three general points that must be borne in mind in carrying out this experiment: (1) that the ATP-electron transfer activity be maximal because of the absence of (a) reaction products such as ADP and phosphate; (b) reagents hydrolyzing high-energy intermediates such as magnesium or uncoupling agents;

(2) that the cytochrome should be in a reduced state blocked from the oxidizing power of molecular oxygen either by anaerobiosis or by a suitable inhibitor of cytochrome oxidase; (3) that the respiratory chain must initially contain both oxidized and reduced components, since for every oxidation there must be a reduction. Succinate-linked pyridine nucleotide can be used as the acceptor of the reducing equivalents from cytochromes from other parts of the chain. Under these conditions the efficiency of the ATP-electron transfer reaction is high, the ATP/ e value being 3 or less. Thermodynamically the phosphate potential required for cytochrome oxidation suggests that ATP can interact with redox couples of the respiratory chain as individuals without supplying a phosphate potential corresponding to the complete span from pyridine nucleotide to cytochrome oxidase.

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Discussion

ARNON: Is it a fair inference from your talk that the mechanism of this reaction (the reduction of DPN by succinate with the aid of ATP) offers a way to study the mechanism of electron transfer but that the reaction as such is of no physiological significance? Do you ascribe any physiological significance to this type of reaction at the cellular level?

CHANCE: I suppose that photosynthesis may be a physiological event, probably the one to which you were referring, and might be of some importance here, and have some comments to make on that tomorrow, particularly on the possibility that light-induced cytochrome responses observed in anaerobic photosynthetic bacteria may be due to photo-produced ATP. Professor Lindberg referred this morning to the reversal of electron transfer into pyridine nucleotide that has a probable physiological implication. Active transport by reversed electron transfer has been considered for nearly a decade.

ARNON: I should add that I was specifically excluding photosynthesis from my question.

AZZONE: What type of mitochondria did you use in your oligomycin experiments?

CHANCE: Pigeon-heart mitochondria.

AZZONE: Why do you think addition of oligomycin inhibits the succinate-induced pyridine nucleotide reduction? Is it not possible to generate the energy necessary for DPN reduction merely by succinate oxidation? My second question is: have you tested the effect of dinitrophenol in your system where you get the cytochrome oxidation after addition of ATP in anaerobiosis?

CHANCE: The answer to your first question is no. Our reaction involves the oligomycin-sensitive steps of Fig. 11. Suitably prepared pigeon-heart mitochondria have an absolute requirement for ATP for succinate-linked reduction of DPN. The answer to the second question is that we have tested 2,4-dinitrophenol and find it to block the ATP-activated reduction of DPN.

AZZONE: As Dr. Ernster will report later oligomycin, at least in liver mitochondria, does not inhibit the succinate-induced pyridine nucleotide reduction. This in our opinion means that, in the presence of oligomycin, liver mitochondria can still synthesize high energy intermediate(s) and that these intermediate(s) can provide the energy required for DPN reduction. Thus in this system there is no requirement for externally added ATP.

With regard to the second question: you know that we have done some experiments in collaboration with Dr. Klingenberg (*Nature, Lond.*, **188**, 552 (1960)) where we have found that dinitrophenol does not inhibit the ATP-induced pyridine nucleotide reduction.

Obviously it was more difficult to observe the ATP effect in our system because the ATP-induced reduction was counter-balanced by the dinitrophenol-induced oxidation of the mitochondrial pyridine nucleotide. Thus your system is perhaps more suited for testing the effect of uncouplers since in anaerobiosis the stimulating effect of dinitrophenol on electron transport is abolished.

CHANCE: We may use oligomycin and 2,4-dinitrophenol to block the ATP-

transfer reactions, and to define the path by which energy is coupled to the reversal of the electron transfer reactions.

SINGER: I would like to ask Dr. Chance why he seems to prefer the diaphorase of the ketoglutaric oxidase complex as being the agent responsible for the reduction of pyridine nucleotide rather than the one of the respiratory chain, since, in the first place, it is commonly believed that there is a spatial separation between these enzymes and, therefore, the mechanism of electron transport between these two systems would not be very obvious. In the second place, the partial inhibition by amylal presents another difficulty, since the ketoglutaric system is not known to be amylal-sensitive.

CHANCE: I completely agree with Dr. Singer, but wanted to point out that there are flavins which had been demonstrated to reduce DPN.

FRENKEL: Can you tell from your difference spectrum whether the DPN is free or enzyme-bound? Dr. Kaplan has informed me that the reduction of enzyme-bound DPN may require appreciably less free energy than the reduction of free DPN.

CHANCE: That is a very interesting observation. The intra-mitochondrial DPNH is bound but it requires about two ATPs for each DPN reduced so considerable energy is required. Its potential may indeed be higher than -300 millivolts but its surely not zero.

ARNON: I would still like to come back to Dr. Chance's comment on the possible significance of this reaction in non-photosynthetic cells.

CHANCE: I thought I had answered that question. This morning Prof. Lindberg referred to some very interesting possibilities where thyroxine might interact with the reduction of DPN and I think this is certainly an example of how this would be a pathway of production of reducing power in the cell, which could be under hormonal control.

SLATER: Dr. Chance's explanation of his results on the basis of reversal of the respiratory chain is very feasible. I am not completely sure that that is the only possible explanation of his results, but this is something I do not think we can possibly go into now. My first question follows on from what Dr. Singer asked. Which flavoprotein do you think you are studying when you are following the flavoprotein spectrum? The second question relates to the very interesting anaerobic experiment with dithionite and ATP where you get DPN reduced and cytochrome *c* oxidized; what is the stoichiometric relationship between the amount of DPN reduced and cytochrome *c* oxidized?

CHANCE: The answer to the first question is that we don't know for sure, because the flavins are unfortunately summed by measurement at $465\text{ m}\mu$, but the amount of flavin which is involved in this pathway is the major portion of the flavin which one observes spectroscopically. Two types of answer are available to your second question. We added $5.6\text{ }\mu\text{moles}$ ATP and we found a total oxidized a_3 , a , c and flavin of 1.5 one-electron equivalents to be oxidized. In other experiments the DPN reduction was slightly in excess of the cytochrome oxidation, but we have not included Q oxidation because it is hard to assay it quantitatively when we add ATP. We find that DPN is reduced faster and flavin oxidized faster than cytochrome *c*. This is apparently the couple into which energy can be put most easily.

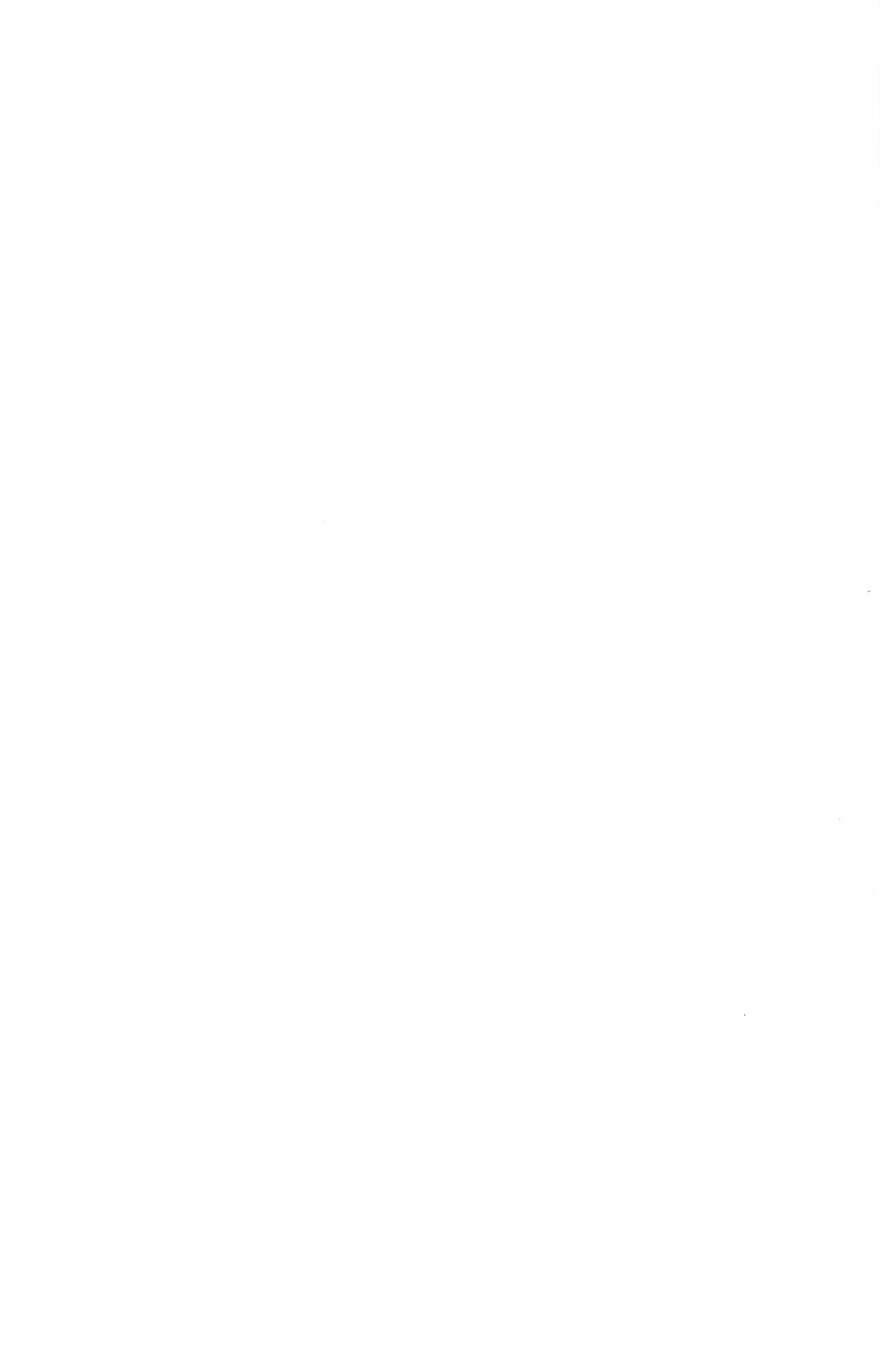
KLINGENBERG: We are also investigating the effects of ATP on the respiratory chain. This research started with the ATP-dependent DPN reduction. The latest results, which I shall show later in the afternoon, have shown that ATP can also interact at the cytochrome region under aerobic conditions. We can induce respiratory control and by this get a cross-over point. Cytochrome *b* is further reduced and cytochrome *c* is further oxidized. We do not see a further oxidation of flavoprotein. Flavoprotein, cytochrome *b* and DPN are all reduced on the addition of ATP, and cytochrome *c*, in a very fast reaction, is oxidized. We believe that this signifies the interaction of ATP at the phosphorylation step between cytochrome *b* and cytochrome *c* and the skeletal muscle mitochondria are thus brought from a slightly uncoupled state to a coupled state by the addition of ATP.

CHANCE: Just a question. Is oxygen present?

KLINGENBERG: Yes.

CHANCE: Well that is very interesting, because it is much more difficult to reverse electron transfer in the cytochrome region when oxygen is present than when oxygen is not present.

KLINGENBERG: This was at the initiation of state 4.



Function of Flavoenzymes in Electron Transport and Oxidative Phosphorylation*†

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Questions concerned with reaction pathways and mechanisms by which flavoenzymes link substrate oxidation to the terminal respiratory chain currently occupy an important position in the research field of mitochondrial electron transport and oxidative phosphorylation. In the present paper two topics relevant to these problems are discussed.

In the first section, data relating to the problem of involvement of quinone reductases, and in particular of vitamin K reductase, in the mitochondrial oxidation of reduced pyridine nucleotides will be presented. Cogent evidence will be put forward that the dicoumarol-sensitive flavo-protein, described by Martius and collaborators [1-4] under the name phyloquinone reductase or vitamin K reductase, does not participate in the main pathway of the mitochondrial oxidation of DPNH. It does constitute the major pathway of direct oxidation of TPNH in the mitochondria, and presumably in the cell. Dr. Conover in this Symposium will present data bearing on this latter point [5].

Recently the concept was developed in our laboratory [6, 7] that the aerobic oxidation of succinate in mitochondria involves an investment of high-energy phosphate, i.e. a type of "activation reaction". The basic lines of evidence underlying this concept will be summarized by Dr. Azzone later this afternoon [8]. Some recent data bearing on the relation of this activation mechanism to the phenomenon of the succinate-linked reduction of mitochondrial pyridine nucleotides, described some time ago by Chance and Hollunger [9, 10] and by Klingenberg and co-workers [11, 12], are the subject of the second section of this paper.

* This work has been supported by grants from the Swedish Cancer Society and the Swedish Medical Research Council.

† The following abbreviations are used: AcAc, acetoacetate; AMP, ADP, ATP, adenosine-5'-mono, di-, and tri-phosphate; DCPIP, 2,6-dichlorophenolindophenol; DPN, DPNH, TPN, TPNH, oxidized and reduced di- and triphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate; P_i, inorganic orthophosphate.

DT Diaphorase: properties and functional aspects

PROPERTIES, AND COMPARISON WITH VITAMIN K REDUCTASE

In 1958, the occurrence of an abundant diaphorase activity in the soluble cytoplasm of rat liver was detected in our laboratory [13, 14]. The enzyme catalyzed the reduction of 2,6-dichlorophenolindophenol by DPNH and TPNH at equal rates, and therefore we decided to call it "DT diaphorase". Early this year [15], we reported the partial purification of the enzyme and some of its properties. These can be summarized as follows: (1) The enzyme is a flavoprotein with a very high turnover number, of the order of ten millions. (2) It reacts at equal maximal rates with DPNH and TPNH, but its affinity for TPNH is slightly higher than for DPNH. (3) Besides various dyestuffs and ferricyanide, a number of naphtho- and benzoquinones can serve as electron acceptors, but not vitamin K₁, or any long-chain substituted quinones. (4) The enzyme is strongly inhibited by dicoumarol, and the inhibition is not competitive with regard to the electron acceptor. (5) It is inhibited by sulphhydryl reagents; and (6) by thyroxine and related compounds. (7) It is activated by bovine serum albumin, which increases both the maximum velocity of the enzyme and its affinity for its substrates. (8) Although the enzyme is present in both mitochondria and microsomes, it is most abundant in the soluble cytoplasmic fraction.

Several of these properties resembled those of various bacterial and plant quinone reductases described by Wosilait and associates [16-18],* as well as those of the vitamin K or phyloquinone reductase of Martius [1-4]. However, DT diaphorase clearly differed from Martius's enzyme in that it did not react at any appreciable rate with vitamin K₁, whereas this compound is the only electron acceptor specified in papers published between 1954 and 1959 by Martius and collaborators.

This situation markedly changed a few weeks ago. In a paper which has just appeared, Märki and Martius [21] now report properties of vitamin K reductase which differ in several important respects from those they previously reported. Moreover the newly reported properties of the enzyme strongly resemble those of DT diaphorase. A brief summary of this development (which escaped recognition in the Märki and Martius paper) is shown in Table I. In fact, except for its insensitivity to -SH reagents and to thyroxine, vitamin K reductase now reveals almost identical properties with those of DT diaphorase, and therefore, we are strongly inclined to conclude that the two enzymes are identical. During the last two years, a considerable amount of information has accumulated in our laboratory

* A similar quinone reductase from dog liver has recently been described by Wosilait [19]. The isolation of a DT diaphorase-like flavoenzyme from brain tissue has been briefly reported by Giuditta and Strecker [20].

concerning the cellular function of D'T diaphorase. This may enable us now to examine critically the role of vitamin K reductase, which, as is well known, has been postulated by Martius [3, 4] to constitute the exclusive pathway of reduced pyridine nucleotide oxidation, and of oxidative phosphorylation, in normal, intact mitochondria. The experimental data to be presented have been obtained in collaboration with Dr. T. E. Conover and Mr. L. Danielson.

TABLE I

COMPARISON OF VITAMIN K REDUCTASE AND D'T DIAPHORASE

Vitamin K Reductase (Martius <i>et al.</i> , 1954-59 [1-4])	D'T Diaphorase (Ernster <i>et al.</i> , 1960 [15])	Vitamin K Reductase (Märki and Martius, 1960 [21])
Flavoenzyme (turnover number, 1.2×10^6).	Flavoenzyme (turnover number, $\sim 10^7$).	Flavoenzyme (turnover number, 7×10^5).
Reacts with DPNH and TPNH at equal rates.	Reacts with DPNH and TPNH at equal rates, but affinity slightly higher for TPNH.	Reacts with DPNH and TPNH at equal rates, but affinity slightly higher for TPNH.
Reacts specifically with vitamin K ₁	Reacts with dyestuffs, ferricyanide, various naphtho- and benzo- quinones, but <i>not</i> with vitamin K ₁ and other long-chain substituted quinones.	Reacts with dyestuffs, ferricyanide, various naphtho- and benzo- quinones, but <i>not</i> with vitamin K ₁ and other long-chain substituted quinones.
Strongly inhibited by dicoumarol.	Strongly inhibited by dicoumarol.	Strongly inhibited by dicoumarol.
Inhibited by thyroxine.	Inhibited by thyroxine (and related compounds).	Not inhibited by thyroxine.
Not inhibited by <i>p</i> - chloromercuribenzoate.	Inhibited by <i>p</i> -chloro- mercuribenzoate. Activated by bovine serum albumin.	Not inhibited by <i>p</i> - chloromercuribenzoate. Activated by bovine serum albumin.
Present in mito- chondria.	Present in mitochondria and microsomes, but bulk in soluble cytoplasm.	Present in mitochondria, but bulk in soluble cytoplasm.

RELATION TO MITOCHONDRIAL RESPIRATORY CHAIN

When intact rat liver mitochondria were incubated with glutamate, and under conditions allowing optimal rates of respiration and phosphorylation, addition of 5×10^{-6} M vitamin K₃ or 10^{-6} M dicoumarol had no effect on the rate of oxygen consumption (Table II). However, when respiration

was blocked by the addition of 1 mM amytal, it could be completely restored by vitamin K_3 and was then strongly inhibited by dicoumarol. The vitamin K_3 -induced respiration was, in accordance with the observations of Colpa-Boonstra and Slater [23], sensitive to antimycin A. The antimycin A inhibition could be overcome to some extent by adding cytochrome *c* (not shown).

From these findings it was concluded that in intact liver mitochondria there exist two pathways of antimycin A-sensitive DPNH oxidase, of which only one, characterized by a sensitivity to amytal, functions under

TABLE II

EFFECT OF VITAMIN K_3 AND VARIOUS INHIBITORS ON THE RESPIRATION OF MITOCHONDRIA IN THE PRESENCE OF AMYTAL

(from Conover and Ernster [22])

Additions	μ atoms oxygen
None	5.49
Vitamin K_3	5.57
Dicoumarol	5.32
Amytal	0.22
Amytal + vitamin K_3	5.25
Amytal + vitamin K_3 + antimycin A	1.57
Amytal + vitamin K_3 + KCN	1.18
Amytal + vitamin K_3 + dicoumarol	1.13

The complete system contained per Warburg vessel: 10 μ moles glutamate, 20 μ moles tris buffer (pH 7.4), 20 μ moles orthophosphate (pH 7.4), 4 μ moles $MgCl_2$, 2 μ moles adenosine triphosphate, 24 μ moles glucose, an excess of yeast hexokinase, 50 μ moles sucrose, and mitochondria from 200 mg. rat liver. The amounts of the additions were as follows: 5×10^{-3} μ mole vitamin K_3 , 10^{-3} μ mole dicoumarol, 1.0 μ mole amytal, 1 μ g. antimycin A, and 1.0 μ mole KCN. Final volume, 1.0 ml. Temperature 30°. Reading begun after 6 min. thermoequilibration. Time measured, 20 min.

normal conditions. Another pathway, characterized by a sensitivity to dicoumarol and proceeding probably via DT diaphorase, is not functioning normally in the terminal oxidation of DPNH, because it lacks a link to the cytochrome system, but it can be brought into reaction by adding an artificial link such as vitamin K_3 .

The phosphorylation arising from the vitamin K_3 -induced respiration in the amytal-blocked system yielded a P/O ratio that was about one unit lower than that of the normal system (Table III). However, the P/O ratio obtained with succinate as substrate also was lowered under these conditions. In other words, it cannot be decided with the evidence presently available, whether or not the vitamin K_3 -induced by-pass of the amytal-sensitive site involves the loss of a phosphorylation.

TABLE III

ESTERIFICATION OF PHOSPHATE DURING THE OXIDATION
OF SUBSTRATE IN THE PRESENCE OF AMYTAL AND VITAMIN K₃

(Conover and Ernster unpublished)

Experimental conditions as in Table II. Substrates were added in a final concentration of 0.01 M.

Substrate	P/O ratio			
	None	Vitamin K ₃	Amytal	Vitamin K ₃ + amytal
glutamate	2.63	2.46	—	1.45
β -OH-butyrate	2.14	1.40	—	1.04
succinate	1.78	1.65	1.53	1.19

The antimycin A-sensitivity of the vitamin K₃-induced respiration in the amytal-blocked system indicated that the electrons mediated by vitamin K₃ may enter the respiratory chain at the level of cytochrome *b*. This point could be tested with the double-beam spectrophotometer,

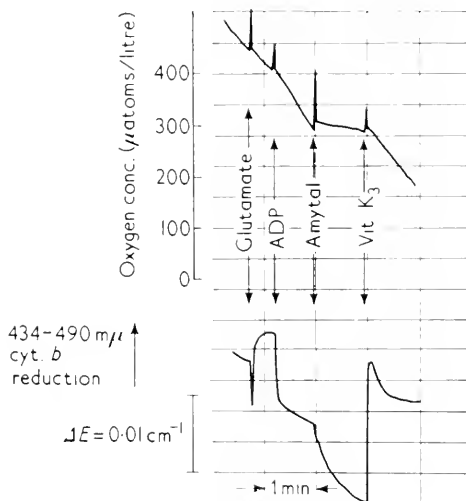


FIG. 1. Effect of amytal and vitamin K₃ on the oxygen consumption and on the reduction of cytochrome *c* during the oxidation of glutamate (Conover and Ernster, unpublished). The medium contained 0.01 M triethanolamine buffer, pH 7.4, 0.01 M P_i, pH 7.4, 0.004 M MgCl₂, 0.065 M KCl, and 0.1 M sucrose. Mitochondria from 200 mg. rat liver were used. The amounts of additions were as follows: 2.0 µmoles L-glutamate, 0.8 µmole ADP, 2.0 µmoles amytal, and 0.04 µmole vitamin K₃. Final volume, 1.2 ml. Temperature, 25°.

kindly placed at our disposal by Dr. M. Klingenberg in Marburg. As shown in Fig. 1, the restoration of respiration by vitamin K_3 in the amytal-blocked system was accompanied by an abrupt increase of the light absorption difference at 434–490 $m\mu$, indicative of a reduction of cytochrome *b*.

SEPARATION OF DT DIAPHORASE AND DPNH OXIDASE

In order further to fortify the concept that DT diaphorase does not participate in the main DPNH oxidase pathway, an attempt was made to separate the two systems. This proved possible by exposing liver mitochondria to disruption by a rapidly rotating Super-Thurrax blender, followed by differential centrifugation, essentially according to the procedure employed by Kielley and Kielley [24] in their studies of mitochondrial ATPase. The procedure results in three submitochondrial fractions: a soluble fraction, a light pellet, and a heavy pellet.

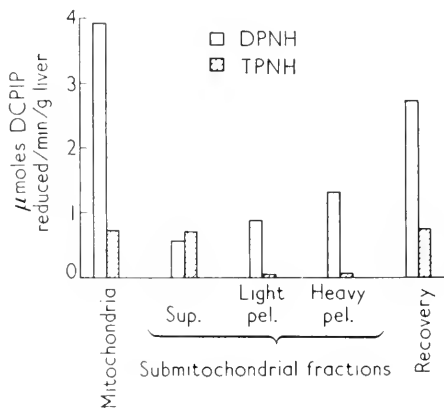


FIG. 2. Diaphorase activities of submitochondrial fractions prepared according to Kielley and Kielley (1953) (from Danielson, Ernster, and Ljunggren [25]).

In examining the DPNH and TPNH diaphorase activities of these fractions it was found (Fig. 2) that the soluble fraction contained virtually the entire TPNH diaphorase activity of the original mitochondria, accompanied by an equal DPNH diaphorase activity, whereas the two pellets exhibited only DPNH diaphorase activities. Moreover, the activities found in the soluble fraction were markedly activated by Tween and strongly inhibited by dicoumarol (both of these properties are characteristic of DT diaphorase), whereas the activities of the pellets were not influenced by these agents (Table IV). The selectivity of the inhibition by dicoumarol between soluble and pellet DPNH diaphorase activities is illustrated in Fig. 3. This treatment of mitochondria thus resulted in a selective solubilization of DT diaphorase.

TABLE IV

PROPERTIES OF DIAPHORASE ACTIVITIES OF SUBMITOCHONDRIAL FRACTIONS PREPARED ACCORDING TO KIELLEY AND KIELLEY (1953)

For experimental details, see Danielson, Ernster and Ljunggren [25].

Submitochondrial fraction	Additions	Diaphorase activity μ moles DCPIP reduced/ min. g. liver	
		DPNH	TPNH
Supernatant	none	0.28	0.30
	8 mg. Tween	0.70	0.71
	8 mg. Tween + 10^{-6} M dicoumarol	0.05	0.04
Pellet (light)	none	0.50	0.008
	8 mg. Tween	0.50	0.038
	8 mg. Tween + 10^{-6} M dicoumarol	0.46	0.006

The light sediment exhibited a DPNH oxidase activity, as shown in Table V. This was sensitive to amytal and antimycin A. When cytochrome *c* was added, the respiration was increased, and the stimulated respiration was only partially inhibited by these agents. Thus this pellet seems to

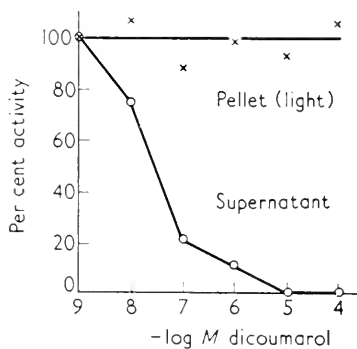


FIG. 3. Effect of dicoumarol on diaphorase activities of submitochondrial fractions prepared according to Kielley and Kielley (1953). Activities measured by using DPNH as substrate and DCPIP as hydrogen acceptor. For experimental details, see Danielson, Ernster, and Ljunggren [25].

contain both the "internal" type of DPNH oxidase of mitochondria, and the "external" type of DPNH cytochrome *c* reductase (cf. [26, 27]). As Professor Lindberg reported earlier in this session [28], the two systems may also be distinguished by their different degrees of sensitivity to desaminothyroxine.

Using the "internal" DPNH oxidase system, that is, the light pellet *without* supplementation with cytochrome *c*, it was found that the amytal-block now could *not* be by-passed by added vitamin K_3 (Fig. 4). However,

TABLE V

PROPERTIES OF DPNH OXIDASE ACTIVITY OF LIVER MITOCHONDRIAL FRAGMENTS PREPARED ACCORDING TO KIELLEY AND KIELLEY (1953)

(Ernster, Danielson and Conover, unpublished)

The test system contained submitochondrial particles ("light pellet") from 200 mg. liver, 0.1 mM DPNH, 0.02 M phosphate buffer, pH 7.5, and where indicated, 1 mM amytal, 0.8 μ g./ml. antimycin A, 0.33 mM KCN, 0.01 mM cytochrome *c*, in a final volume of 3 ml. The oxidation of DPNH was followed at 340 m μ in a recording Beckman DK2 spectrophotometer.

Addition	μ moles DPNH oxidized/min./g. liver	
	Without cytochrome <i>c</i>	With cytochrome <i>c</i>
None	0.16	0.46
Amytal	0.03	0.23
Antimycin A	0.03	0.22
KCN	0.01	0.02

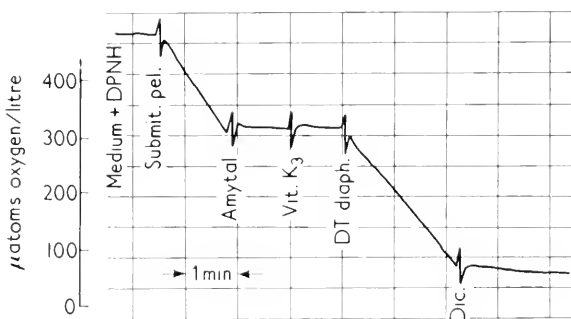


FIG. 4. Requirement of DT diaphorase for the vitamin K_3 -mediated oxidation of DPNH by mitochondrial fragments in presence of amytal (Conover and Ernster, unpublished). The medium contained 19 μ moles orthophosphate (pH 7.5) and 2 mg. serum albumin in 1.0 ml. Submitochondrial fragment preparation from 1 g. of rat liver was used. The amounts of the additions were as follows: 1.0 μ mole DPNH, 2.0 μ moles amytal, 0.005 μ mole vitamin K_3 , purified DT diaphorase capable of reducing 1 μ mole DCPIP per min., and 0.03 μ mole dicoumarol. Final volume, 1.3 ml. Temperature, 20.

the by-pass could be achieved by adding a purified sample of DT diaphorase.

QUINONE SPECIFICITY

These early studies were carried out using vitamin K_3 as the only quinone. Then Dr. Conover made the somewhat surprising observation that, although DT diaphorase can react with a number of both naphtho-

and benzoquinones, only vitamin K₃, out of a great number of quinones tested, was able to carry out the above described by-pass of the mitochondrial amytal-sensitive site. This phenomenon is illustrated in Table VI. It can be seen that there was a clear requirement for both the naphthoquinone skeleton and the 2-Me substituent when the mitochondrial respiratory chain served as terminal electron acceptor. This requirement

TABLE VI

REQUIREMENT FOR 2-METHYL-1,4-NAPHTHOQUINONE STRUCTURE IN MEDIATION OF ELECTRON TRANSPORT BETWEEN D_F DIAPHORASE AND MITOCHONDRIAL RESPIRATORY CHAIN

(from Conover and Ernster [22], and unpublished data)

Quinone*	Relative activity			
	As terminal electron acceptor	As electron mediator to		
		Cytochrome <i>c</i>	"Cytochrome <i>b</i> " [†]	
		System I	System II	
Vitamin K ₃ (2-Me-1,4-naphthoquinone)	100 [†]	100	100	100
1,4-naphthoquinone	93	140	5	15
1,2-naphthoquinone	72	87	0	19
<i>p</i> -benzoquinone	174	0	0	3
2-Me-benzoquinone	159	6	0	3
2,6-diMe-benzoquinone	168	20	0	2
CoQ ₀ (2-Me-5,6-dimethoxybenzoquinone)	155	22	0	3
Vitamin K ₁	0	0	2	2
Vitamin K ₂	0	0	1	2
CoQ ₁₀	0	0	0	2

* The quinones were used in final concentrations of 33 or 67 μ M as terminal acceptors, and 8.33 or 10 μ M as mediators.

[†] This activity was about 1.5 times that obtained with DCPIP as acceptor.

[‡] System I: Intact mitochondria, glutamate, amytal.

System II: Submitochondrial DPNH oxidase (Kielley and Kielley, light pellet), TPNH, purified D_F diaphorase, KCN.

was valid both for intact mitochondria and for the reconstructed submitochondrial system, thus eliminating effects due to permeability barriers. With purified cytochrome *c*, coupling occurred with both 2-Me substituted and non-substituted naphthoquinone, and to some extent also with 2-Me benzoquinones. The general rule that we presently envisage, after having tested a large collection of quinones, is schematically illustrated in Fig. 5.

It is obvious that any type of quinone specificity involved in the function of DT diaphorase is a specificity on the acceptor and not on the donor side. In other words, vitamin K may be the specific coupler of DT diaphorase to the cytochrome system in the living cell; however, *not* because DT diaphorase requires vitamin K specifically but because cytochrome *b* does. Whatever the reason for this requirement may be, it is clear that the name "vitamin K reductase" for the flavoenzyme as such is hardly adequate.

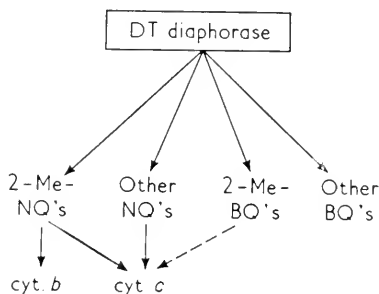


FIG. 5. Quinone-catalyzed coupling between DT diaphorase and cytochromes. NQ = naphthoquinone; BQ = benzoquinone; Me = methyl.

CONCLUSIONS AND COMMENTS

Figure 6 summarizes in a schematic form our conclusions as to the relation of DT diaphorase to the main pathway of mitochondrial DPNH oxidation. It seems to be clear from the data presented above that the amytal-sensitive, DPNH specific pathway represents the main, if not exclusive, route of DPNH oxidation in normal, intact rat-liver mitochondria. The dicoumarol-sensitive DT diaphorase, which very probably is identical with Martius and collaborators' vitamin K reductase, is present in these mitochondria without any apparent functional link to the terminal electron transport system. In order to establish such a link, the external supply of a catalytic amount of vitamin K_3 (or any other 2-methylnaphthoquinone without a long carbon-chain substituent in the 3-position) is needed for electrons from DT diaphorase to enter the respiratory chain at or before the level of cytochrome *b*. Alternatively, DT diaphorase can be coupled to the respiratory chain by way of naphthoquinones without a 2-methyl substituent, and to some extent also by way of 2-methylbenzoquinones. In these cases, external cytochrome *c* is required in addition to the quinone, and the entrance of the electrons takes place beyond the antimycin A sensitive site of the chain, probably at the level of cytochrome *a*.

There are only two further brief comments I would like to add to these conclusions. First, it should be pointed out that although Martius's vitamin K reductase does not seem to participate in the main pathway of

DPNH oxidation in liver mitochondria this does not imply that Martius's original idea [29] of the participation of vitamin K in the main respiratory chain need to be abandoned. Indications of a specific role of vitamin K in DPN-linked respiration and phosphorylation have been reported, both in fractionated bacterial systems [30] and in U.V.-irradiated liver-mitochondrial preparations [31, 32]. Even if this evidence is only circumstantial, its validity is not influenced by the present conclusions. It may well be that bound vitamin K does participate as an electron carrier in the amytal-sensitive, main pathway of mitochondrial DPNH oxidation,

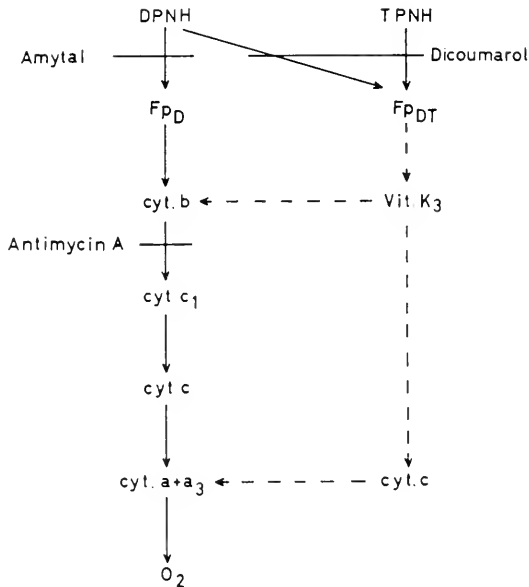


FIG. 6. Relation of DT diaphorase to the main pathway of DPNH oxidation of intact liver mitochondria. F_{pD} = DPNH diaphorase; F_{pDT} = DT diaphorase. Dotted arrows indicate pathways involving externally added carriers.

located, tentatively, between the DPNH diaphorase and cytochrome *b*. Such a possibility may actually find some indirect support in our data, which indicate that cytochrome *b* might require a 2-methylnaphthoquinone as a specific electron donor. Pertinent to this possibility may also be the preliminary findings, illustrated in Table VII, that the DPNH diaphorase reaction of the Kielley and Kielley preparation reveals a marked sensitivity to amytal when measured with 2-methyl-substituted quinones as electron acceptors, whereas it exhibits only a slight amytal sensitivity with non-substituted quinones. Thus, the role of vitamin K in respiration and phosphorylation deserves further consideration.

A second point of interest is that of the well-known uncoupling effect

of dicoumarol on mitochondrial oxidative phosphorylation [29]. The rational standpoint in view of the present conclusions would seem to be that this effect is independent of the inhibitory effect of dicoumarol on DT diaphorase. Alternatively, one could think that DT diaphorase, although not taking part in the main pathway of terminal electron transport, might

TABLE VII

CAPACITY OF VARIOUS QUINONES TO ACT AS TERMINAL ELECTRON ACCEPTORS IN SUBMITOCHONDRIAL DPNH OXIDASE, AND THE AMYTAL SENSITIVITY OF THESE REACTIONS

(Ernster, Danielson and Conover, unpublished)

The quinones were added in final concentrations of 0.04 mM. Oxygen uptake in these systems was blocked with 0.3 mM cyanide.

Terminal electron acceptor	Relative activity	% inhibition by 2 mM amytal
Oxygen	1.0	81
2-Me-1,4-naphthoquinone	1.3	52
2-Me-benzoquinone	2.7	64
2-Me-5,6-dimethoxybenzoquinone (CoQ ₀)	2.8	40
1,4-naphthoquinone	3.1	12
1,2-naphthoquinone	9.9	18
<i>p</i> -benzoquinone	9.5	5

play some accessory role, such as a regulation of the redox-state of the mitochondrial pyridine nucleotides, during coupling of respiration to phosphorylation. However, the fact that certain tissues, e.g. pigeon liver [21], seem to contain very little or none of the dicoumarol-sensitive flavoenzyme, and still exhibit a highly active phosphorylation, would seem to impose serious obstacles to a consideration of this alternative.

Activation of succinate oxidation and succinate-linked reduction of DPN

OBSERVATIONS WITH HIGH-ENERGY PHOSPHATE-DEPLETED MITOCHONDRIA

I wish to begin the second section of my report by quoting a finding that Dr. Löw and I described in 1955 [33]. At that time we found (Fig. 7) that rat liver mitochondria exposed to ageing in a phosphate-containing medium lost some of their succinoxidase activity in the course of the ageing process. The decrease was of a transitional character; upon prolonged ageing, the mitochondria resumed their original succinoxidase activity. We found, moreover, that the decreased succinoxidase activity could be

restored to its original level by the addition of ATP. From these and other findings, the hypothesis was advanced that mitochondria, above a certain level of structural organization, require ATP for oxidizing succinate, and that, at this transitional stage of ageing, the mitochondria had already lost their endogenous ATP while they still possess part of their organized feature.

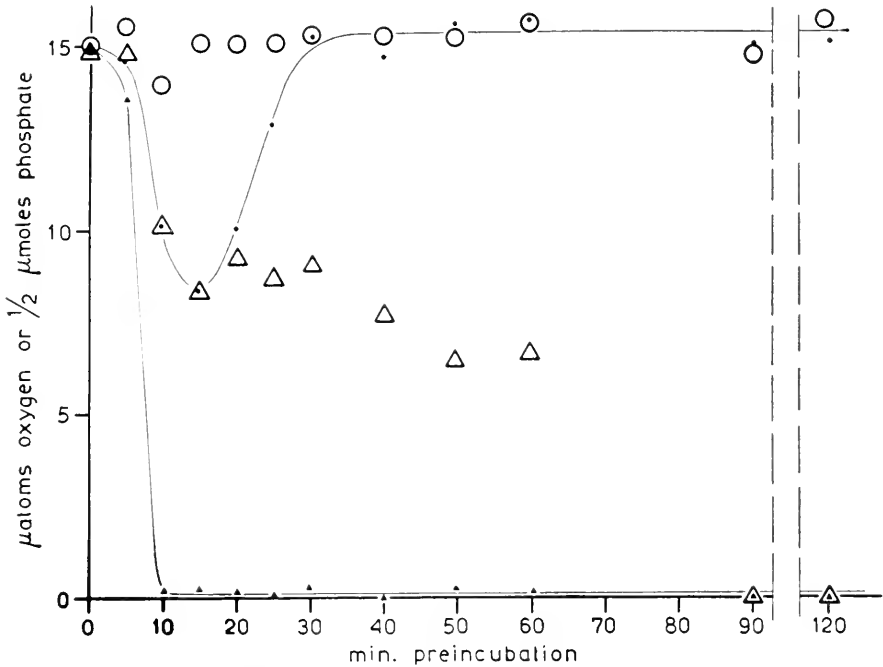


FIG. 7. Effect of ageing on succinoxidase activity of rat liver mitochondria (from Ernster and Löw [33]). The continuous lines indicate oxygen (upper line) and phosphate (lower line) uptake in *absence* of ATP and Mg⁺⁺; the large circles indicate oxygen uptake, and the large triangles phosphate uptake, in *presence* of ATP and Mg⁺⁺.

These findings were almost forgotten when last winter Dr. Azzone in our laboratory made the interesting observation [6] that rat liver mitochondria after preincubation in the presence of 2 mM arsenate and 0.04 mM dicoumarol (or 0.1 mM dinitrophenol) for a period of 3 to 4 min. displayed a strongly inhibited succinoxidase activity, which could be greatly enhanced by added ATP. In extending this observation (which is illustrated in the upper part of Fig. 8) it was found [7] that the decrease of the succinoxidase activity was correlated with an exhaustion of the endogenous high-energy phosphate content of the mitochondria; and conversely, that any means of pretreating mitochondria so as to deplete them of high-energy

phosphate, provided that it caused no irreversible damage of their structural integrity, led to a decrease of the succinoxidase activity. Furthermore, what was only anticipated in 1955 (and even questioned later), namely, that the observed inhibition was not due to an accumulation of oxaloacetate, could now be ascertained by rigorous experimental means [7, 8].

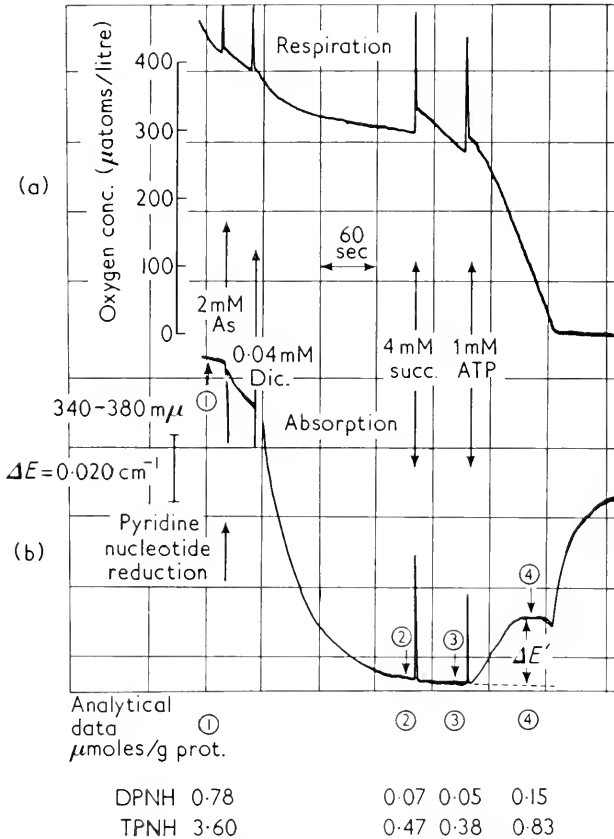


FIG. 8. Effect of ATP on succinate oxidation and pyridine nucleotide reduction in rat liver mitochondria preincubated with arsenate and dicoumarol (from Azzone, Ernster, and Klingenberg [34]).

From work along these lines the concept was developed that in intact liver mitochondria, the aerobic oxidation of succinate involves an activation reaction by means of high-energy phosphate. Since this mechanism was visualized as involving the formation of a high-energy intermediate in the respiratory chain at the level where the electrons derived from succinate enter the terminal respiratory chain, it seemed conceivable that this intermediate might also be involved in the endergonic reduction of the

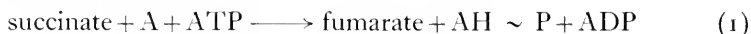
mitochondrial DPN by succinate, earlier described by Chance and Hollunger [9, 10] and by Klingenberg *et al.* [11, 12]. It was therefore of interest to investigate whether the ATP-induced activation of succinate oxidation in the high-energy phosphate depleted mitochondria was reflected in an increased level of DPNH.

In this part of our investigation, Dr. Azzone and I had the privilege to benefit by the hospitality and collaboration of Dr. Martin Klingenberg at Marburg. A typical result of our experiments, a detailed account of which is being published elsewhere [34], is shown in the lower part of Fig. 8. As can be seen, the ATP-induced stimulation of the aerobic oxidation of succinate in the arsenate-dicoumarol depleted mitochondria was paralleled by an increase of the 340–380 absorption difference, indicative of the reduction of pyridine nucleotides. Similar results were obtained when dicoumarol was replaced by 10^{-4} M dinitrophenol. Differential analytical data, given at the bottom of the figure, reveal that the increase was due to a major part to the reduction of TPN, and to a minor part to the reduction of DPN. Admittedly, the observed steady-state levels of the reduced pyridine nucleotides were not particularly high, about 10 and 30% of the total contents of DPN and TPN, respectively. On the other hand, the system contained a fully uncoupling concentration of dicoumarol (or dinitrophenol), this causing a maximal flux of electrons towards oxygen; the levels of DPNH and TPNH found may thus actually represent the maximal values obtainable in an uncoupled system.

However, the main importance of these findings in our opinion is the very fact that an ATP-dependent reduction of pyridine nucleotides by succinate could occur at all in the presence of a fully uncoupling concentration of dicoumarol or dinitrophenol; or in other words, that a high-energy intermediate at the level of the respiratory chain could be formed at the expense of ATP in spite of the uncoupled state of the oxidative phosphorylation system. This seems to imply a serious challenge to those proposed schemes of oxidative phosphorylation ([35], [36], [26]; cf. [37] for review) which involve the participation of non-phosphorylated high-energy intermediates at the level of the electron transport chain, and according to which uncoupling agents act by disconnecting the interaction of this intermediate with inorganic phosphate and ADP. On the other hand, the present finding is in agreement with, and even lends some support to, the hypothesis promulgated by our group [38–40] that phosphorylated reduced electron carriers are the primary high-energy intermediates. According to this hypothesis, uncouplers are visualized as acting by preventing inorganic phosphate from becoming activated in connection with the energy-yielding oxidative step, and are thus not expected to interfere with the reversal of this reaction.

On the basis of the above findings, the following simple reaction scheme

has been proposed [7] for the activation of succinate oxidation and the succinate-linked reduction of mitochondrial DPN:



where A stands for the electron carrier whose reduction by electrons derived from succinate requires an investment of high-energy phosphate. Depending on the nature of A, Reaction (1) or (2), or both of them, may be sum-reactions involving several steps. It may be, thus, that A is succinic dehydrogenase, in which case Reaction (1) is a one-step reaction, and Reaction (2) a several-step one, composed of a transfer of P from AH ~ P to the reduced diaphorase-flavin, and a subsequent reversal of the first respiratory chain phosphorylation. It may also be that A is the diaphorase-flavin; then, Reaction (1) may be composed of a reduction of succinic

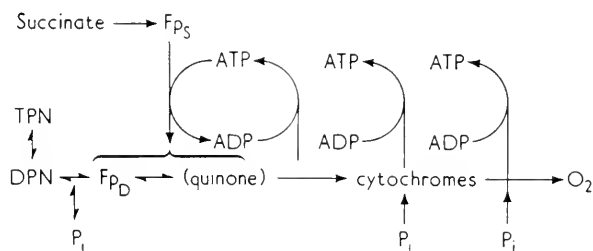
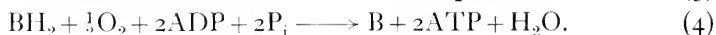
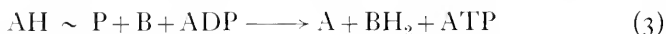


FIG. 9. Hypothetical scheme of the functional link of succinic dehydrogenase to the terminal electron transport system (from Azzone, Ernster, and Klingenberg [34]). Fp_D = DPNH diaphorase; Fp_s = succinic dehydrogenase.

dehydrogenase by succinate, followed by an ATP-requiring, phosphorylative reduction of A by the reduced succinic dehydrogenase. A third alternative may be that A is a quinone, in which case both Reactions (1) and (2) are composed of two steps; Reaction (1) by a reduction of succinic dehydrogenase by succinate, followed by an ATP-dependent, phosphorylative reduction of A by the reduced succinic dehydrogenase; and Reaction (2) by a transfer of P from AH ~ P to the reduced diaphorase-flavin, followed by a reversal of the first respiratory chain phosphorylation. In any case, AH ~ P must be of such a nature that its reoxidation by the subsequent carrier (B) along the respiratory chain be connected with a regeneration of ATP, and moreover, that the further oxidation of the resulting BH_2 by molecular oxygen still can give rise to two net phosphorylations; otherwise, the aerobic oxidation of succinate in mitochondria could not result in a P/O ratio of 2. These reactions may then be written as:



A schematic illustration of these concepts is found in Fig. 9.

ENDERGONIC REDUCTION OF ACETOACETATE BY
SUCCINATE IN LIVER MITOCHONDRIA*

Up to the present, the succinate-linked reduction of mitochondrial DPN has exclusively been studied by measuring the increase in the steady-state level of endogenous DPNH, ensuing upon the addition of succinate and/or ATP. One obvious limitation of this system, emerging from the above reasoning, may be that any increase in the DPNH level that one observes is a resultant of two, independent, reaction capacities; on one hand, the capacity of the reaction(s) feeding in electrons from succinate into the respiratory chain (Reaction 1 in the above formulae), and on the other, the capacity of the chain of reactions by which these electrons are transferred from their point of entrance to oxygen (Reactions 3 and 4). Clearly, if the latter capacity is equal to or exceeds the former, no increase in the level of DPNH may be expected to occur. This situation may render the reproducibility of the observations, e.g. from one tissue or one set of conditions to another, dependent on irrelevant circumstances, and in particular, it may render unrealistic a quantitative evaluation of the number of high-energy bond equivalents required for the reduction of one molecule DPN by succinate. Moreover, using the above test system, no conclusive evidence has yet been presented that the reducing equivalents appearing in DPNH actually originate from succinate and not from some endogenous substrate, the oxidation of which has been facilitated, in a secondary manner, by succinate and/or ATP.

To overcome these difficulties, it was felt desirable to devise a system in which the DPN reduced by succinate was continuously reoxidized by suitable means, e.g. by way of the reversal of a DPN-linked dehydrogenase reaction. The reversal of the β -hydroxybutyric dehydrogenase reaction, consisting of a reduction of acetoacetate to β -hydroxybutyrate, was considered to be convenient for this purpose, since this is the only known reaction by which free acetoacetate can be metabolized in rat liver mitochondria. It was in fact found that when isolated rat liver mitochondria were incubated in the presence of succinate and acetoacetate under aerobic conditions and in the absence of phosphate acceptor, there occurred a substantial disappearance of acetoacetate which was linear with time and strictly dependent on the presence of succinate (Fig. 10). Furthermore, the acetoacetate reduction was completely inhibited by 2 mM amytal, indicating that it involved an electron transfer between the site of entrance of electrons from succinate into the respiratory chain and DPN. Replacement of succinate by malate, with or without amytal, resulted only in an

* The studies reported in this and the following section have been conducted in collaboration with Drs. G. F. Azzone and E. C. Weinbach.

insignificant reduction of acetoacetate, probably because of the unfavourable equilibrium of the malate + acetoacetate \rightleftharpoons oxaloacetate + β -hydroxybutyrate system. This finding, together with the amytal-sensitivity of the succinate-linked reduction, thus clearly eliminated the possibility that malate originating from succinate rather than the latter itself might constitute the reducing agent for acetoacetate.

As could be expected from the great positive difference in redox potential between the succinate/fumarate and DPNH/DPN couples (cf. [10]), the succinate-linked reduction of acetoacetate in the present system was strictly dependent on an active oxidative phosphorylation coupled to

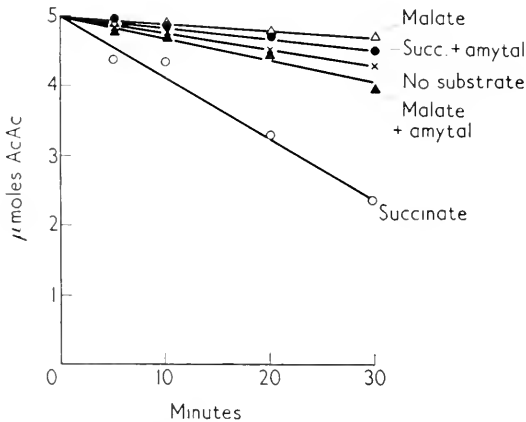


FIG. 10. Reduction of acetoacetate by succinate in rat liver mitochondria (Azzone, Ernster, and Weinbach, unpublished). Each flask contained: mitochondria from 150 mg. liver, 20 mM glycylglycine buffer, pH 7.5, 8 mM $MgCl_2$, 62 mM sucrose, 25 mM KCl, 5 mM P_i , 5 mM acetoacetate, and, when indicated, 10 mM succinate, 10 mM L-malate, 2 mM amytal, in a final volume of 1 ml. Incubation at 30°. Acetoacetate determined according to Walker [41].

the terminal oxidation of succinate. Accordingly, as shown in Fig. 11, the acetoacetate reduction was abolished by dinitrophenol. It may be noticed that half-inhibition was reached at a concentration of about 5×10^{-6} M, which is considerably below that required for a corresponding depression of the oxidative phosphorylation. This finding is in agreement with the data of Chance and Hollunger [15], from their spectrophotometric studies of the succinate-linked reduction of endogenous DPN. Since dinitrophenol is known to abolish respiratory control at a concentration lower than that needed for an actual depression of the phosphorylating capacity [42, 43], these data indicated that the reduction of acetoacetate by succinate in the present system was dependent not only on an active oxidative phosphorylation but also on a state of respiratory control, the latter allowing

the maintenance of adequate levels of high-energy intermediates needed for the endergonic reduction of DPN by succinate.

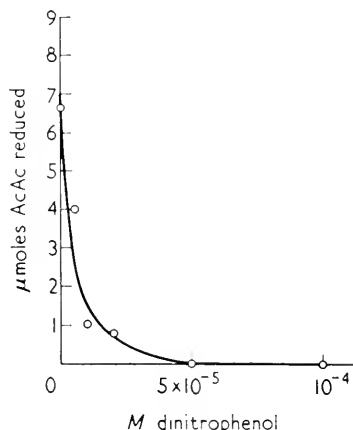


FIG. 11. Inhibition of succinate-linked reduction of acetoacetate by 2,4-dinitrophenol (Azzone, Ernster, and Weinbach, unpublished). Each flask contained: mitochondria from 400 mg. liver, 10 mM succinate, 5 mM acetoacetate, and dinitrophenol as indicated, in a final volume of 2 ml. Other conditions as in Fig. 10. Time of incubation, 20 min.

TABLE VIII

INFLUENCE OF Mg^{++} AND OF TERMINAL PHOSPHATE ACCEPTOR ON THE SUCCINATE-LINKED REDUCTION OF ACETOACETATE

(Azzone, Ernster and Weinbach, unpublished)

Each Warburg flask contained: mitochondria from 250 mg. liver, 20 mM glycyglycine buffer, pH 7.5, 62 mM sucrose, 50 mM KCl, 10 mM succinate, 5 mM acetoacetate, 15 mM P_i , and where indicated, 15 mM AMP, 1 mM ATP, 15 mM glucose, and an excess of yeast hexokinase, in a final volume of 2 ml. Gas phase, air; in centre well, 0.2 ml. 2 M KOH; temperature, 30°. Time of incubation, 16 min.

Additions	8 mM Mg^{++}			No Mg^{++}		
	-JAcAc (μmoles)	Oxygen (μatoms)	P O	-JAcAc (μmoles)	Oxygen (μatoms)	P O
—	2.6	4.6		0.3	8.7	
AMP	0	11.0	1.63	0	11.3	1.79
ATP, hexokinase, glucose	0.1	11.2	1.82			

This concept was further emphasized by the findings recorded in Table VIII. It has been demonstrated first by Baltcheffsky [44] that incubation of rat liver mitochondria in the absence of Mg^{++} abolishes the

requirement for phosphate acceptor in maintaining a high rate of respiration, without parallel loss of the actual phosphorylating capacity. Such a "loose-coupling" of phosphorylation from respiration, as revealed by an increased respiratory rate in the absence of phosphate acceptor, was in the present case accompanied by an almost complete abolition of the acetoacetate reduction. The phosphorylating capacity of the Mg^{++} -deficient system (measured with AMP as terminal phosphate acceptor) was the same as that found in the presence of Mg^{++} . As anticipated, no acetoacetate reduction took place in the presence of the terminal phosphate

TABLE IX

INFLUENCE OF Mg^{++} ON THE SUCCINATE-LINKED REDUCTION OF ACETOACETATE UNDER VARIOUS CONDITIONS

(Azzone, Ernster and Weinbach, unpublished)

Experimental conditions as in Fig. 13, except that mitochondria from 300 (in Experiment 2, 200) mg. liver were added per flask.

Experiment No.	Additions	No Mg^{++}	8 mM Mg^{++}
		- Δ AcAc (μ moles)	
1	—	1.4	4.0
	ATP (1 mM)	4.0	3.8
	EDTA (2 mM)	1.0	4.4
	ATP (1 mM), EDTA (2 mM)	1.0	3.9
2	—	0.6	2.4
	ATP (1 mM)	2.7	2.2
	Oligomycin A (1 μ g. ml.)	0.7	3.2
	ATP (1 mM), oligomycin A (1 μ g./ml.)	3.0	3.2
3	—	0.4	3.9
	NaF (10 mM)	3.3	
	P_i omitted	0.6	4.2
	NaF (10 mM), P_i omitted	5.9	

acceptor because of the continuous removal of the high-energy bonds generated in the respiratory chain. These findings substantiate the above conclusion that "loosely-coupled" respiration cannot give rise to succinate-linked DPN-reduction even though the phosphorylating capacity of the mitochondria remains intact.

Addition of ATP to the Mg^{++} -deficient system restored the acetoacetate reduction (Table IX), but this effect was counteracted by EDTA, suggesting that it was dependent on endogenous Mg^{++} . The ATP effect was not abolished by oligomycin A, and thus cannot be due to a supply of energy to the respiratory chain. Furthermore, 10 mM sodium

fluoride gave a similar effect. This ATP-effect may be analogous to the effect of ATP in inducing DPN-reduction by succinate, recently observed by Chance and Hagihara [45] in aged pigeon-heart mitochondria.

It was briefly indicated above (Table IX) that oligomycin A did not inhibit (in fact even slightly stimulated) the succinate-linked reduction of acetoacetate in the present system. As shown in Fig. 12, this compound was also able to restore efficiently acetoacetate reduction when this was suppressed because of the presence of a terminal phosphate acceptor (in this case ADP, hexokinase and glucose). Oligomycin A has been shown by

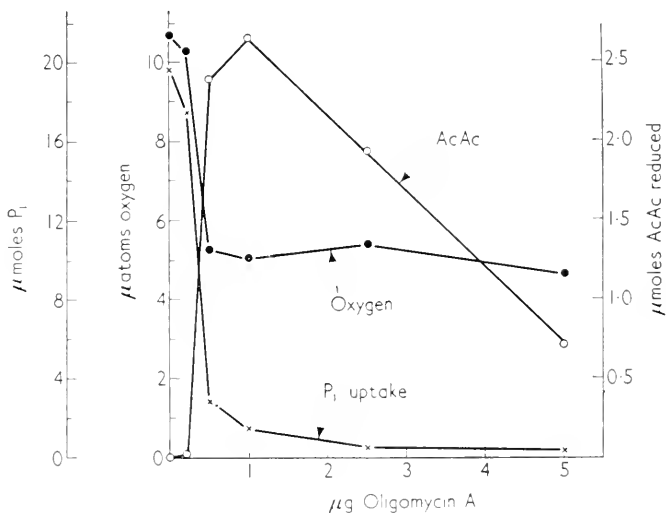


FIG. 12. Effect of oligomycin A on succinate-linked reduction of acetoacetate in presence of terminal phosphate acceptor (Azzone, Ernster, and Weinbach, unpublished). Experimental conditions as in ATP-hexokinase-glucose system in Table VIII.

Lardy *et al.* [46] to inhibit mitochondrial respiration under phosphorylating conditions but not if the phosphorylation is abolished by dinitrophenol; in extending these studies we found that oligomycin A only inhibits tightly-coupled, but not "loosely-coupled", respiration. Furthermore, according to Lardy *et al.* [46], oligomycin A also strongly inhibits the mitochondrial P_i -ATP exchange and dinitrophenol-induced ATPase reactions. From these observations, oligomycin A appears to act by blocking the transfer of phosphate from the primary high-energy bonds to ADP. This mode of action fits logically with the present findings that oligomycin A removed the phosphate acceptor effect from the succinate-linked reduction of acetoacetate. What is more interesting, however, is that the transfer of energy from the sites of the succinate-linked phosphorylations to the site

where it is utilized for DPN-reduction is apparently not affected by the oligomycin A-block. Accepting the above mode of action of oligomycin A, this would mean, either that this transfer can take place *directly*, without the intermediary of ATP, or that it proceeds via a fraction of intramitochondrial ATP which is not available to hexokinase and glucose and whose interaction with the primary high energy intermediates is not blocked by oligomycin A.

Based on the conclusion, reached above, that "loosely-coupled" respiration could not contribute energetically to the succinate-linked DPN-reduction, it was considered possible to estimate the stoichiometry of the DPN-reducing system by measuring the difference in rate of succinate oxidation, observed in the presence and absence of added acetoacetate. It may be assumed that the respiration observed in the

TABLE X

STIMULATION OF SUCCINATE OXIDATION DUE TO REDUCTION OF
ACETOACETATE IN MITOCHONDRIA IN CONTROLLED STATE

(Azzone, Ernster, and Weinbach, unpublished)

Experimental conditions as in Table VIII, except that mitochondria from 400 mg. liver and 25 mM P_i were added per flask. All flasks contained 8 mM MgCl₂. Oligomycin A, when present, was added in a concentration of 1 μg./ml.

Additions	O ₂ consumption, μatoms		μmoles AcAc	
	With AcAc	Without AcAc	ΔO ₂	Reduced
None	9.78	7.68	2.10	4.7
Oligomycin A	9.11	6.71	2.40	5.7
ATP, hexokinase, glucose	19.3	18.5	0.8	0
Oligomycin A, ATP, hexokinase, glucose	10.13	7.17	2.96	5.4

absence of both phosphate acceptor and acetoacetate, being "loosely-coupled", cannot contribute energy to the reduction of DPN; and consequently, that addition of acetoacetate to the phosphate acceptor-free system would result in an increase of the respiratory rate, in a phosphate acceptor-like manner, to the extent it "trapped" energy from the respiratory chain by reoxidizing endergonically reduced DPN. Data presented in Table X are the mean values of duplicate runs and are reasonably precise. It is seen that addition of acetoacetate to the phosphate acceptor-free system resulted in an increase in oxygen consumption by 2.10 μatoms, and this was accompanied by a disappearance 4.7 μmoles of acetoacetate. In the presence of oligomycin A (which slightly stimulated both the

respiratory increase and the acetoacetate reduction) the corresponding values were $2.40 \mu\text{atoms}$ and $5.7 \mu\text{moles}$, respectively. Addition of ATP, hexokinase and glucose, which abolished acetoacetate reduction, also significantly diminished the respiratory stimulation due to acetoacetate, to the value of $0.8 \mu\text{atom}$; and, finally, addition of oligomycin A to the hexokinase-glucose system restored both respiratory stimulation and acetoacetate reduction to about their original levels, $2.96 \mu\text{atoms}$ and $5.4 \mu\text{moles}$, respectively. Thus, in all three cases where acetoacetate reduction occurred, there occurred a respiratory stimulation as well, the rate of which was approximately $0.5 \mu\text{atom oxygen per } \mu\text{mole acetoacetate reduced}$. Assuming a P/O ratio of 2 for the aerobic oxidation of succinate to fumarate, this implies a ratio of one high energy bond equivalent per

TABLE XI

EFFECT OF RESPIRATORY CHAIN INHIBITORS ON SUCCINATE-LINKED
REDUCTION OF ACETOACETATE IN RAT LIVER MITOCHONDRIA

(Azzone, Ernster, and Weinbach, unpublished)

In each flask: 5 mM acetoacetate, 25 mM succinate, 62 mM sucrose, 50 mM KCl, 20 mM tris buffer, pH 7.5, 8 mM MgCl₂, mitochondria from 400 mg. liver. When indicated: 5 mM ATP, 2 mM amytal, 1.25 $\mu\text{g. ml.}$ antimycin A, 1.25 $\mu\text{g. ml.}$ oligomycin A, 0.5 mM KCN. Final volume, 2 ml. Gas phase, air. Temperature, 30°. Time of incubation, 20 min.

Additions	- JAcAc (μmoles)	
	-ATP	+ATP
None	3.4	3.2
Antimycin A	0	0
Cyanide	0	0
N ₂ as gas phase	0	0
Oligomycin A	3.9	4.0

molecule of acetoacetate reduced. This value is in agreement with that envisaged by the reaction mechanism for the succinate-linked reduction of DPN, discussed above (cf. Reactions 1 and 2, and Fig. 9), and is considerably lower than those previously arrived at by Chance and Hollunger [10] and by Chance [47].

As shown in Table XI, the succinate-linked reduction of acetoacetate in the present system was completely abolished by the respiratory inhibitors, antimycin A and cyanide, as well as in anaerobiosis. Added ATP, which had no effect on the aerobic system, did not remove these inhibitions. Hence, in contrast to the succinate-linked reduction of DPN in the high-energy phosphate-depleted mitochondria (cf. Fig. 10), the succinate-linked reduction of acetoacetate in non-depleted mitochondria appears to

require an intramitochondrially generated supply of high-energy compounds. As will be indicated below, this is probably due to compartmentation phenomena in the intact mitochondrion.

It was of special interest to establish whether the inhibitory effect of antimycin A was merely due, like those of cyanide and anaerobiosis, to a general block of the energy-generating system, or whether this compound inhibited the succinate-linked reduction of DPN *per se*. The latter conclusion has recently been reached by Chance and Hollunger [10] and led them to postulate that the succinate-linked DPN-reduction involves cytochrome *b*. The reaction scheme proposed by us (cf. Fig. 9) is not compatible with such a conclusion.

TABLE XII

PHOSPHORYLATION COUPLED TO THE ANTIMYCIN A INSENSITIVE OXIDATION OF SUCCINATE BY FERRICYANIDE IN RAT LIVER MITOCHONDRIA

(Azzone, Ernster and Weinbach, unpublished)

In each flask: mitochondria from 300 mg. liver, 50 mM KCl, 20 mM glycylglycine, pH 7.5, 12.5 mM P_i , 8 mM $MgCl_2$, 10 mM succinate, 20 mM ferricyanide, 0.5 mM KCN, 1 mM ATP, 15 mM glucose, hexokinase, and, when indicated, 1 μ g./ml. antimycin A. Final volume, 2 ml. Incubation at 30° for 20 min.

	Without antimycin A	With antimycin A
μ moles $(Fe(CN)_6)^{3+}$ reduced	16.0	18.5
μ moles P_i esterified	5.5	4.2
$P/2e^-$	0.69	0.46

Copenhaver and Lardy [48] reported in 1952 that oxidation of succinate in rat liver mitochondria with ferricyanide as terminal electron acceptor in the presence of cyanide gave rise to a phosphorylation with a $P/2e$ ratio of 0.6, and that both the oxidation and the coupled phosphorylation were insensitive to antimycin A. These findings were now confirmed (Table XII)* and, using this system, it could be shown that the succinate-linked

* Subsequent to the studies of Copenhaver and Lardy [48], Pressman [49] reported that, in his hands, antimycin A did inhibit the oxidation of succinate by ferricyanide. In an attempt to explore the reason for this discrepancy, we found (cf. also *Discussion*, p. 168) that the antimycin A-sensitivity of this system is dependent on the P_i/ATP ratio prevailing in the incubating medium; high concentrations of P_i favour insensitivity, and high concentrations of ATP favour sensitivity, to antimycin A. Furthermore, both P_i and ATP were found greatly to stimulate ferricyanide reduction. These findings are strikingly parallel to those reported by Hatefi [50] concerning the reduction of coenzyme Q in beef heart mitochondria and the antimycin A-sensitivity of this reaction.

TABLE XIII

INSENSITIVITY OF SUCCINATE-LINKED REDUCTION OF ACETOACETATE TO ANTIMYCIN A

(Azzone, Ernster, and Weinbach, unpublished)

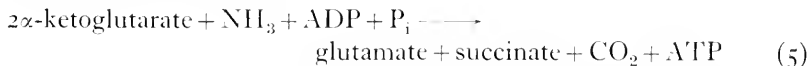
In each flask: mitochondria from 300 mg. liver, 50 mM KCl, 20 mM glycylglycine, pH 7.5, 12.5 mM P_i , 8 mM $MgCl_2$, 10 mM succinate, 3 mM acetoacetate, and, where indicated, 20 mM ferricyanide, 1 μ g. ml. antimycin A and 0.5 mM KCN. Final volume, 2 ml. Incubation at 30° for 20 min.

Additions	μ moles AcAc reduced
None	2.6
KCN	0.3
KCN + $(Fe(CN)_6)^{3+}$	2.0
KCN + $(Fe(CN)_6)^{3+}$ + Antimycin A	2.4

reduction of acetoacetate was completely insensitive to antimycin A (Table XIII).

AMINATIVE REDUCTION OF α -KETOGLUTARATE BY SUCCINATE

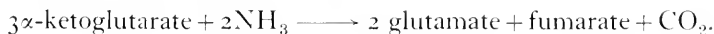
Before terminating my report, let me briefly show you some preliminary data obtained with another dehydrogenase, glutamic dehydrogenase, as a trapping system for the DPNH generated by succinate-linked DPN-reduction. This system was very similar to that used by Hunter and Hixon in 1949 [51] for the demonstration of α -ketoglutarate-linked substrate-level phosphorylation. It consisted of liver mitochondria incubated under anaerobic conditions in the presence of α -ketoglutarate and ammonia. In this system, as Hunter and Hixon have shown, the following reaction takes place:



However, unlike Hunter and Hixon's system, the present one was not supplemented with a terminal phosphate acceptor. It was thus expected that the ATP and succinate formed in the α -ketoglutarate oxidation would give rise to a reduction of DPN, after which the DPNH so generated would reduce another molecule of α -ketoglutarate (+ ammonia) to glutamate. This reaction sequence may be written as follows:



The net reaction of Reactions 5-7 is:



Since Reaction 6, but not Reaction 5, is inhibited by amytal, it could be expected that, if Reactions 6 and 7 were occurring, the disappearance of α -ketoglutarate would be diminished in the presence of amytal. The data in Table 14 show that, indeed, there occurred an amytal-sensitive α -ketoglutarate utilization; however, unexpectedly, this was dependent on the presence of externally added ATP. As anticipated, on the other hand, the amytal-sensitive part of the α -ketoglutarate utilization could be completely abolished by hexokinase and glucose. Further data, not included in Table XIV, show that the CO_2 production, in contrast to the α -ketoglutarate utilization, was not influenced by amytal. All these data are thus consistent with the above reaction sequence.

TABLE XIV

ATP-DEPENDENT AMINATIVE REDUCTION OF α -KETOGLUTARATE BY
SUCCINATE IN ANAEROBIC MITOCHONDRIA

(Azzone, Ernster, and Weinbach, unpublished)

In each flask: 0.05 M KCl, 0.02 M glycylglycine buffer, pH 7.5, 8 mM MgCl_2 , 10 mM P_i , 5 mM NH_4Cl , 5 mM α -ketoglutarate, mitochondria from 150 mg. liver. When indicated: 2 mM amytal, 5 or 1 mM ATP (without or with hexokinase-glucose). Final volume, 2 ml. Temperature 30°. Time of incubation, 20 min. Gas phase, N_2

Additions	μ moles α -ketoglutarate consumed	
	Without amytal	With amytal
None	3.2	3.4
ATP	6.3	3.7
ATP, hexokinase, glucose	3.8	3.9

However, regarding the requirement for external ATP, it is obvious that, from the point of view of energy-transfer, this system is fundamentally different from the succinate-linked reduction of acetoacetate. The role of ATP cannot be that of merely "tightening" the mitochondrial structure (as in the case of the Mg^{++} -deficient acetoacetate system, cf. Table IX), since, in the present case, oligomycin A counteracted the ATP effect. It was also found in preliminary experiments that ATP could be replaced by catalytic amounts of AMP. This eliminates the possibility that the α -ketoglutarate-linked phosphorylation might not be able to furnish energy to the succinate-linked reduction of DPN. Whether this discrepancy in ATP requirement between the acetoacetate and α -ketoglutarate systems is due to the different dehydrogenases, β -hydroxybutyric and glutamic, or to the different sites of phosphorylation, respiratory chain and substrate level, involved in the two systems, cannot be decided at this time. In any

case, it is indicative of a complex pattern of compartmentation of energy-transfer routes within the mitochondria. Similar indications have recently been obtained in our laboratory along other lines of approach [52, 53].

CONCLUSIONS

The main conclusions of the second section of this paper may be summarized as follows:

1. In rat liver mitochondrial preparations depleted of high-energy phosphate by preincubation with arsenate and dicoumarol or dinitrophenol, the oxidation of succinate is greatly stimulated by ATP. Parallel to the respiratory stimulation, the mitochondrial pyridine nucleotides become reduced to a slight but significant extent. It is concluded that the ATP-induced activation of succinate oxidation and the ATP-induced reduction of DPN by succinate involve a common high-energy intermediate, which consists of a phosphorylated, reduced electron carrier, and whose formation at the expense of ATP and succinate is not inhibited by uncoupling concentrations of dinitrophenol and dicoumarol. The reduction of DPN by succinate is thought to involve a reversal of the DPN-flavin-linked oxidative phosphorylation, and its extent in a respiring system is consequently a resultant of the rate at which electrons derived from succinate enter the respiratory chain and the rate at which these electrons are transferred from their site of entrance towards oxygen.

2. Intact liver mitochondria incubated under aerobic conditions in the absence of phosphate acceptor catalyze a reduction of acetoacetate to β -hydroxybutyrate, coupled to the oxidation of succinate to fumarate. The reaction, which provides conclusive evidence for a substantial transfer of hydrogen from succinate to mitochondrial DPN, is completely inhibited by amytal, as well as by low concentrations of dinitrophenol, addition of terminal phosphate acceptor, or omission of Mg^{++} . The phosphate acceptor effect is removed by oligomycin A, which presumably acts by blocking the transfer of phosphate between the primary high-energy intermediates and ADP. In the Mg^{++} -deficient system acetoacetate reduction is restored by ATP and by sodium fluoride. The succinate-linked acetoacetate reduction is also suppressed by antimycin A, cyanide, or in the absence of oxygen; under these conditions, the reduction is not restored by added ATP. Ferricyanide, in the presence of cyanide, allows phosphorylation coupled to succinate oxidation, and also restores acetoacetate reduction. Under appropriate conditions, both the coupled phosphorylation and the acetoacetate reduction of the ferricyanide system are insensitive to antimycin A. Respiration with succinate as substrate in the absence of phosphate acceptor is stimulated by acetoacetate and the stimulation corresponds to $0.5 \mu\text{atom oxygen per } \mu\text{mole acetoacetate reduced}$.

From these findings it is concluded that the reduction of acetoacetate by succinate, catalyzed by tightly-coupled liver mitochondria, involves a reversal of the DPN-flavin-linked oxidative phosphorylation; that the energy required for this process is equivalent to one high-energy bond per molecule of acetoacetate reduced; that this energy can be supplied by one or both of the two terminal respiratory chain phosphorylations without the intermediary of extramitochondrial ATP; and that the succinate-linked reduction of mitochondrial DPN does not involve the antimycin A-sensitive site of the respiratory chain.

3. Liver mitochondria under anaerobic conditions catalyze an aminated reduction of α -ketoglutarate to glutamate, coupled to the oxidation of succinate to fumarate, which proceeds at the expense of high-energy phosphate generated in the α -ketoglutarate-linked substrate-level phosphorylation. Some preliminary observations are presented which suggest the existence of a complex pattern of compartmentation of mitochondrial energy-transfer routes.

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Discussion

WILLIAMS: I should like to ask Dr. Ernster why he thinks pyridine nucleotide reduction is necessary in the ATP-activating succinate respiration? I ask this question for two reasons, first: in his own results respiration in fact seemed to be active at a time when little reduction of pyridine nucleotide had taken place; secondly, because in collaborative experiments with Dr. Chance this summer in Philadelphia, we were also able to reactivate the respiration with ATP while seeing essentially no change in the steady states of the pyridine nucleotides. You didn't speculate on the reason why you needed pyridine nucleotide reduction.

ERNSTER: We do not mean that we need pyridine nucleotide reduction. Our point is that the two mechanisms: activation of succinate oxidation and succinate-linked DPN-reduction may be correlated in the sense that, during the initiation of succinate oxidation, a reduced high-energy intermediate is generated at the level of the respiratory chain, and that this intermediate is identical with, or in close relationship to, the high-energy intermediate involved in the first respiratory

chain phosphorylation. Then, depending on how large the electron flux is toward oxygen, you may or may not get a DPN-reduction.

CHANCE: I would like to congratulate Dr. Ernster on the excellent results he has got in spite of the very difficult experimental problem of using acetoacetate reduction as the assay for the intramitochondrial reduction of pyridine nucleotides. First, we agree completely on the antimycin-sensitivity, provided ATP is used as the energy source. Secondly, endogenous substrate is a real factor to be considered. Thirdly it wasn't clear to me in the experiments of ferricyanide reduction whether or not that system had an exogenous ATP requirement. If it had I would have expected it to have shown antimycin-sensitivity.

ERNSTER: In the ferricyanide experiment there was no ATP added and no ATP requirement. These conditions were apparently suited for producing sufficient energy for the reduction of pyridine nucleotide, by way of the phosphorylation occurring between succinic dehydrogenase and ferricyanide. That phosphorylation was not sensitive to antimycin A.

CHANCE: It is possible that we are in agreement on the antimycin A because we could do the ferricyanide experiment in the way that requires ATP.

TABLE I

EFFECT OF P_i AND ATP ON ANTIMYCN A-SENSITIVITY OF
REDUCTION OF FERRICYANIDE BY SUCCINATE

The system contained: mitochondria from 300 mg. rat liver, 100 μ moles KCl, 40 μ moles glycylglycine buffer pH 7.5, 16 μ moles $MgCl_2$, 20 μ moles succinate, 30 μ moles ferricyanide, 1 μ mole KCN, and, when indicated, 1 μ g. antimycin A, in a final volume of 2 ml. Incubation for 20 min. at 30 .

Additions (μ moles)		Ferricyanide reduced (μ moles)	
P_i	ATP	- Antimycin A	+ Antimycin A
—	—	10.3	10.6
15	—	19.8	20.2
60	—	21.9	24.1
—	10	16.5	2.8
15	10	22.3	22.3
60	10	29.7*	28.8*

* Complete reduction.

ERNSTER: Yes, I can see that. Let me show a slide here which illustrates how complicated this system is (Table I). It can be seen that the ferricyanide reduction by succinate may or may not be antimycin A sensitive depending on the concentrations of phosphate and ATP; and furthermore, that both phosphate and ATP stimulate ferricyanide reduction, in an additive manner. I can't explain these data but they do illustrate, I think, the complexity of the system.

Coupling of Reduced Pyridine Nucleotide Oxidation to the Terminal Respiratory Chain

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As was reported previously [9], a soluble enzyme has been isolated which can couple the oxidation of both TPNH and DPNH† to the reduction of a number of dyes and other electron acceptors. This enzyme, which has been called "DT diaphorase" from its lack of specificity towards the pyridine nucleotides, has been found in all cellular fractions examined, but exists predominately in the cytoplasm. The properties of this enzyme and its interaction with various quinones and with the respiratory chain of mitochondria have been studied at some length. In this paper some of these studies will be reported with the hope of drawing a possible inference as to the role of this abundant enzyme in the cell.

The importance of the level of extramitochondrial or cytoplasmic reduced pyridine nucleotide in the control and regulation of metabolism and synthesis has been pointed out by Krebs [14], Dickens [6], and others. The mechanism of the regulation of the levels of these reduced pyridine nucleotides, however, remains incompletely understood. This is particularly true in the case of TPNH, a substance which is essential for cellular synthetic reactions. It is without question that a most important point in the regulation of these levels of reduced pyridine nucleotide is the control of their mitochondrial oxidation. Since the DT diaphorase reacts with both DPNH and TPNH and is abundantly present in the cytoplasm, the question of whether this enzyme functions in the oxidation of cytoplasmic reduced pyridine nucleotide was carefully considered.

If freshly prepared mitochondria from rat liver were incubated with soluble cytoplasm from rat liver prepared by centrifugation at $105\ 000 \times g$

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† Abbreviations: TPNH, reduced triphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate; DCPIP, 2,6-dichlorophenolindophenol.

and with added glucose-6-phosphate and TPN, very little consumption of oxygen was observed, as is shown in Fig. 1. This is due to the fact that, as has been reported previously by Pullman and Racker [16], TPNH, which is formed from the oxidation of glucose-6-phosphate, is not readily oxidized by mitochondria. If vitamin K_3 in low concentration was added, however, there was initiated a rapid oxygen uptake, indicating a rapid

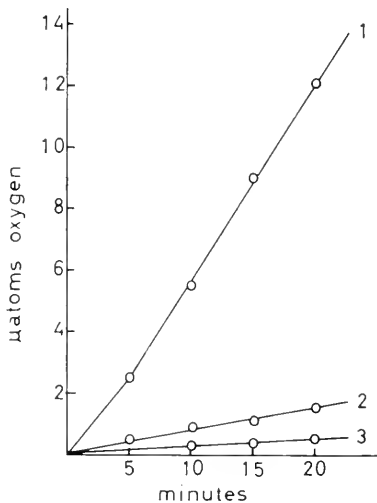


FIG. 1. Oxidation of glucose-6-phosphate by liver mitochondria in the presence of soluble cytoplasm and vitamin K_3 . The complete system contained per Warburg vessel 1.0 μ moles TPN, 20 μ moles glucose-6-phosphate, 50 μ moles tris buffer (pH 7.4), 30 μ moles orthophosphate (pH 7.4), 10 μ moles $MgCl_2$, 5 μ moles adenosine triphosphate, 60 μ moles glucose, an excess of yeast hexokinase, 225 μ moles sucrose, 0.03 μ mole vitamin K_3 , dialyzed supernatant fluid centrifuged at $105\,000 \times g$ from 450 mg. rat liver, and mitochondria from 500 mg. rat liver. Final volume, 3.0 ml. Temperature, 30°. Reading begun after 5 min. thermoequilibration.

1. Complete system.
2. No vitamin K_3 .
3. No mitochondria.

oxidation by the mitochondria of the TPNH formed in the incubation medium.

This respiration was insensitive to Amytal but was inhibited by Antimycin A and cyanide (Table I). Most importantly, it was observed to be sensitive to dicoumarol at concentrations of 10^{-5} M or less, which is a very characteristic property of DT diaphorase [9].

The implication that DT diaphorase was involved in the oxidation of extramitochondrial TPNH in this system was supported by the duplication of this system with isolated enzymes. Mitochondria freshly prepared from

TABLE I

EFFECT OF SOME INHIBITORS ON VITAMIN K₃-STIMULATED OXIDATION OF GLUCOSE-6-PHOSPHATE BY MITOCHONDRIA AND SOLUBLE CYTOPLASM

Additions (+) or omissions (—)	Relative oxygen consumption
Complete system	100
+ 2×10^{-3} M Amytal	109
+ 1 μ g. Antimycin A	39
+ 10^{-3} M KCN	24
+ 10^{-6} M dicoumarol	43
— vitamin K ₃	31

Conditions as in Fig. 1. Respiration of complete system was 10.12μ atoms oxygen/20 min.

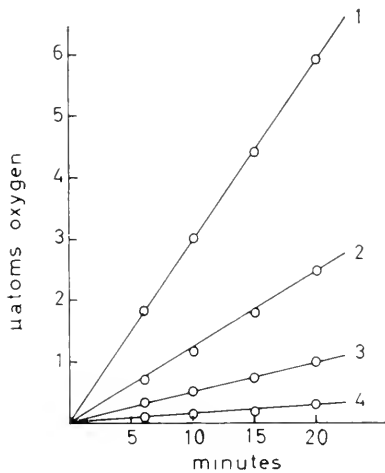


FIG. 2. Mitochondrial oxidation of extramitochondrial TPNH mediated by DT diaphorase and vitamin K₃. The complete system contained per Warburg vessel 0.5μ mole TPN, 20μ moles glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase (Sigma), 20μ moles tris buffer (pH 7.4), 12μ moles orthophosphate (pH 7.4), 4μ moles MgCl₂, 2μ moles adenosine triphosphate, 24μ moles glucose, an excess of yeast hexokinase, 50μ moles sucrose, mitochondria from 200 mg. rat liver, 0.01μ mole vitamin K₃, and an amount of 450-fold purified DT diaphorase (together with 1 mg. serum albumin) capable of reducing 1μ mole vitamin K₃ per min. Final volume, 1.0 ml. Temperature 30°. Reading begun after 6 min. thermoequilibration. [3]

1. Complete system.
2. No DT diaphorase.
3. No DT diaphorase, no vitamin K₃.
4. No mitochondria.

rat liver were incubated in an isotonic buffered medium containing TPN, glucose-6-phosphate, purified Zwischenferment, phosphate, Mg^{++} , ATP, hexokinase, and glucose. As is shown in Fig. 2 the extramitochondrially-generated TPNH was not oxidized under these conditions to any appreciable extent by the mitochondria in agreement with the previous experiment. Addition of vitamin K_3 produced a two- to threefold stimulation of the total respiration. Addition of purified cytoplasmic DT diaphorase [9] to the vitamin K_3 -stimulated system gave a further two- to threefold stimulation of the respiration. This rate of respiration was close to that obtained maximally with succinate or glutamate as substrate and it may represent the limit of the cytochrome system to react with oxygen rather than that of TPNH to react with the cytochrome chain.

TABLE II

EFFECT OF SOME INHIBITORS ON THE MITOCHONDRIAL OXIDATION OF EXTRA-MITOCHONDRIAL TPNH MEDIATED BY DT DIAPHORASE AND VITAMIN K_3

Additions (+) or omissions (—)	Relative oxygen consumption
Complete system	100
+ 2×10^{-3} M Amytal	118
+ 1 μ g. Antimycin A	20
+ 10^{-3} M cyanide	24
+ 10^{-6} M dicoumarol	24
— DT diaphorase, — vitamin K_3	20

Conditions as in Fig. 2. Respiration of complete system was 5.56μ atoms oxygen/20 min [3].

The pattern of inhibition by various inhibitors was identical in this system to that in the previous one (Table II). The inhibition of the respiration by Antimycin A and cyanide is in agreement with the report of Colpa-Boonstra and Slater [2] on the oxidation of reduced vitamin K_3 by mitochondria. This inhibition by Antimycin A suggests the entrance of electrons into the respiratory chain at, or above, the site of Antimycin A inhibition, probably at the level of cytochrome *b*. This is supported by other experiments which are reported elsewhere [8].

As DT diaphorase can reduce a wide variety of quinones tested [8, 9] it was assumed that this stimulation of the oxidation of TPNH was a general property of such quinones. However, as is shown in Table III, it was highly specific for 2-methyl-naphthoquinones, and in particular, for vitamin K_3 . Such a specificity increased the anticipation of a biological role for such a system. While it was recognized that vitamin K_3 is a highly artificial material for a biological system it was felt that possibly some

TABLE III

EFFECT OF VARIOUS QUINONES ON THE MITOCHONDRIAL OXIDATION OF EXTRAMITOCHONDRIAL TPNH IN THE PRESENCE OF DT DIAPHORASE

Additions	Relative oxygen consumption
vitamin K ₃ (2-methyl-1,4-naphthoquinone)	100
1,2-naphthoquinone	17
1,4-naphthoquinone	14
2-hydroxy-1,4-naphthoquinone	14
2-methyl-3-hydroxy-1,4-naphthoquinone	24
<i>p</i> -benzoquinone	15
2-methylbenzoquinone	10
2,6-dimethylbenzoquinone	9
coenzyme Q ₁₀ (2-methyl-5,6-dimethoxybenzoquinone)	14
none	13

Conditions as in Fig. 2. The amount of quinone was 10^{-2} μ mole added in 10 μ l. ethanol. Respiration of the system with vitamin K₃ was 5.20 μ atoms oxygen/20 min. [Conover and Ernster, unpublished].

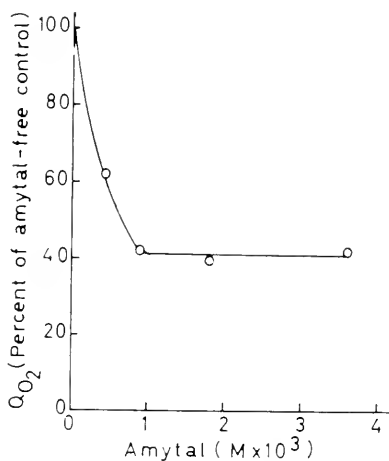


FIG. 3. Effect of Amytal on the respiration of liver slices. Each Warburg vessel contained 50 μ moles glucose, 200 mg. rat liver slices and 2.0 ml. Krebs-ringer-phosphate solution (pH 7.4). Gas phase, oxygen. Temperature, 37°. Time measured, 60 min. [7].

naturally-occurring quinones of a similar type might serve in a similar role in the intact cell.

It was observed by Ernster [7] that there was in liver slices an Amytal-resistant respiration which amounted to about 30–40% of the total observed respiration with glucose as substrate (Fig. 3). This is in contrast to the more recent report of Chance and Hess [1] with ascites tumour cells where the respiration was completely inhibited by Amytal. Investigations were initiated by Mr. Kadenbach in this laboratory on the nature of this Amytal-resistant respiration with particular emphasis on whether DT diaphorase might be involved.

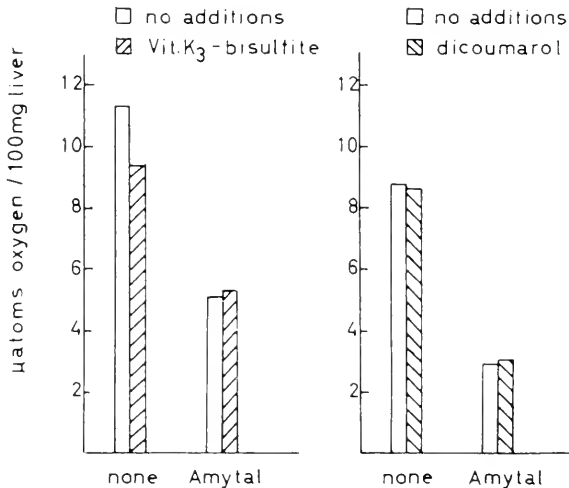


FIG. 4. The effect of vitamin K₃ and dicoumarol on the respiration of liver slices in the presence and absence of Amytal. Each Warburg vessel contained 50 μ moles glucose, 100 mg. wet weight rat liver slices, and 0.8 ml. Krebs-Ringer-phosphate solution containing half the usual concentration of CaCl₂. The amounts of the additions were 2 μ moles Amytal, 10⁻² μ mole vitamin K₃-bisulphite, and 2 \times 10⁻² μ mole dicoumarol. The final volume was 1.0 ml. Gas phase, oxygen. Temperature, 37.5°. Time measured, 60 min.

Preliminary investigation quickly showed that the function of DT diaphorase in either the normal or the Amytal-resistant respiration of rat liver slices with glucose as substrate would be difficult to demonstrate. Figure 4 shows in a simple manner two experiments in which the addition of vitamin K₃-bisulphite had no stimulatory effect on either normal or Amytal-resistant respiration, nor did addition of dicoumarol show any inhibitory effect on respiration in these two conditions.

The water-soluble vitamin K₃-bisulphite was used in these experiments in order to avoid addition of alcohol to the system, since alcohol is

readily used as substrate by the slices. It was shown with the isolated enzyme system that vitamin K_3 -bisulphite may also be used in stimulating the mitochondrial oxidation of TPNH presumably because the bisulphite portion is readily split off.

In view of the various possible oxidative pathways open to the DPNH which would be generated from the oxidation of glucose it was felt that the DT diaphorase system in the rat liver slice might better be demonstrated with a substrate which would specifically generate TPNH.

In Fig. 5 is shown the effect of dicoumarol on the Amytal-resistant respiration of rat liver slices in the presence of citrate as substrate. It can be seen that while there was some stimulation of citrate respiration by

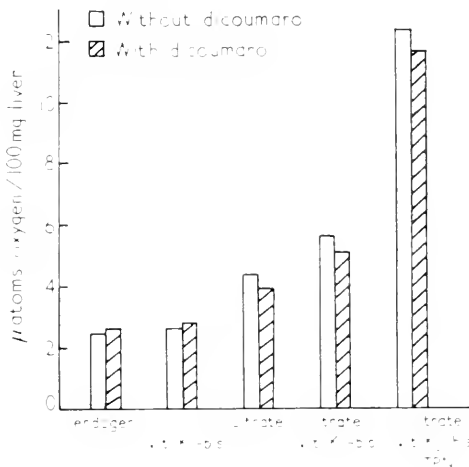


FIG. 5. The dicoumarol sensitivity of the Amytal-resistant respiration of liver slices with citrate as substrate. Conditions as in Fig. 4. The amounts of the additions were $25 \mu\text{moles}$ citrate, $5 \times 10^{-2} \mu\text{mole}$ vitamin K_3 -bisulphite, $1 \mu\text{mole}$ TPN, and $5 \times 10^{-3} \mu\text{mole}$ dicoumarol. Time measured, 60 min. [13].

vitamin K_3 , there was very little dicoumarol sensitivity characteristic of DT diaphorase. It was therefore apparent that unless there was an impermeability of the slice to dicoumarol that there must exist alternative amytal-insensitive pathways of TPNH oxidation. One such possibility considered was a trans-hydrogenation to DPN and subsequent oxidation of the DPNH formed. It has been suggested by various workers [11, 15, 17] that the dehydrogenases which are able to react with both DPNH and TPNH may, in the presence of their substrate, act as transhydrogenases. If this is the case it might be assumed that lactic dehydrogenase constitutes the major portion of the transhydrogenase activity in the cytoplasm.

On this basis the previous experiment was repeated on rat liver slices

which were preincubated with iodoacetate in order to deplete the level of lactate and pyruvate and therefore lower the transhydrogenase activity. It can be seen in Fig. 6 that this preincubation has not changed greatly the pattern of citrate oxidation. Vitamin K_3 still stimulated respiration to some extent. Most striking, however, is that the citrate respiration was now markedly inhibited by dicoumarol.

It would therefore appear that the DT diaphorase system can be demonstrated in the intact cell with liver slices in the presence of added vitamin K_3 under conditions where the transhydrogenase activity of the cytoplasm is low. It is difficult, however, to ascertain from this whether DT diaphorase is able to function in the respiration and oxidation of reduced pyridine nucleotide in normal conditions. Under usual slice conditions, particularly in the presence of Amytal where lactate levels are high, the activity of DT diaphorase may be masked by the transhydrogenase activity.

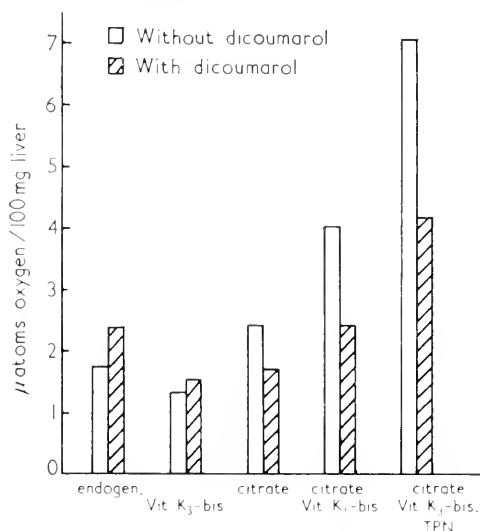


FIG. 6. The dicoumarol sensitivity of the Amytal-resistant respiration of iodoacetate-pretreated liver slices. Conditions as in Fig. 4 except that the slices were preincubated for 20 min. at 37.5° in the presence of 2×10^{-4} M iodoacetate. Time measured, 60 min. [13].

Several of the properties of DT diaphorase would suggest some limitations in its function in the cell. From a Lineweaver-Burk plot for the enzyme it was seen that the Michaelis constants for the enzyme are high; 0.18 mM for DPNH and 0.13 mM for TPNH. Albumin, which activates the enzyme in the purified state, gives a marked lowering of the K_M to 0.08 mM and 0.04 mM respectively. TPNH in both cases has a somewhat lower K_M than has DPNH. It would seem that though the

enzyme has a very high turnover number [9] it requires rather high levels of substrate in order to function efficiently.

This effect may be illustrated by comparing the activity of two types of diaphorase enzymes, the purified DT diaphorase and the D diaphorase of the mitochondrial respiratory chain prepared by extraction of mitochondria with Lubrol W. The diaphorase activity of these two enzymes was compared in the oxidation of both added DPNH and of DPNH generated with a system containing alcohol dehydrogenase and ethanol, which at the pH used has an equilibrium unfavourable to the production of DPNH. As may be seen in Table IV the two diaphorase activities were chosen so as to react at similar rates with added DPNH as substrate. In the alcohol dehydrogenase system D diaphorase could still function efficiently; however, the reaction rate with DT diaphorase was greatly reduced as compared with the activity with added DPNH.

TABLE IV

COMPARISON OF THE ACTIVITIES OF DT DIAPHORASE AND DPNH DIAPHORASE OF MITOCHONDRIA WITH ADDED DPNH AND WITH A DPNH GENERATING SYSTEM

Substrate	Diaphorase activity	
	Purified DT diaphorase	Mitochondrial D diaphorase
	(μmoles DCPIP reduced min.)	
DPNH	0.020	0.017
Ethanol, DPN, alcohol dehydrogenase	0.0024	0.0165

The assay system contained 0.04 mM DCPIP, 0.1 mM DPNH or DPN, 33 mM ethanol, excess alcohol dehydrogenase, 0.33 mM KCN, 0.1% albumin, and 0.05 mM orthophosphate (pH 7.5). Reaction followed by ΔE_{600} . [Ernster, Danielson, and Ljunggren, unpublished].

It seems then that DT diaphorase would function in the cell only when the levels of reduced pyridine nucleotide are high. As Glock and McLean [10] and others [12] have shown this is generally found only in the case of TPNH. It is assumed, therefore, that the function of DT diaphorase is primarily with regard to the TPNH of the cell.

Although the bulk of this enzyme is located in the cytoplasm it can also be extracted from mitochondria [5]. Here the function of the enzyme is perhaps even more obscure than it is in the cytoplasm. Numerous transhydrogenases have been reported which would presumably allow the oxidation of TPNH through the active DPNH oxidase of mitochondria.

As has been reported previously [4, 8] DT diaphorase can be demonstrated in mitochondria by bypassing the site of Amytal inhibition in the

oxidation of pyridine nucleotide-linked substrates by the addition of vitamin K_3 . However, DT diaphorase seems to play no role in the normal respiration of mitochondria. Some coupling of mitochondrial DT diaphorase to the respiratory chain by naturally occurring quinones may occur and may indeed account for the observed TPNH oxidase and cytochrome c -reductase activity in mitochondria; however, the necessity for such a pathway is difficult to comprehend.

In conclusion, it must be said that though a role for DT diaphorase in cellular respiration has been diligently searched for, the evidence of its participation in this role is rather meagre. Figure 7 is recorded with some hesitation as it probably implies more than may actually be the case. It is possible that when TPNH levels are high that DT diaphorase may function as is diagrammatically shown and as has been shown to occur with

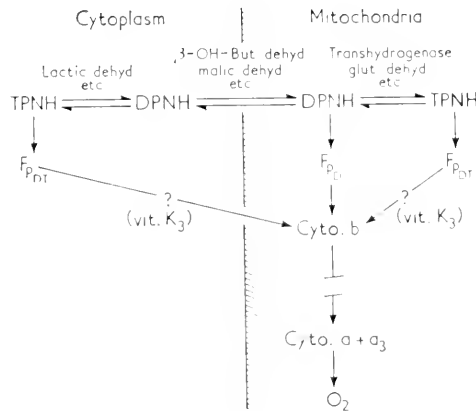


FIG. 7. Some possible pathways of reduced pyridine nucleotide oxidation and its regulation.

added vitamin K_3 . This pathway would require the presence of some natural mediator, presumably of a quinone type, to function between DT diaphorase and the respiratory chain. It may be emphasized that in liver at least it is the absence or unavailability of sufficient amounts of such a low molecular weight mediator that is essential for the maintenance of the high levels of TPNH, rather than the absence of an enzyme which can oxidize TPNH. Similar conclusions have been reached by Wenner [18] in regard to the operation of the glucose-6-phosphate shunt in ascites tumour cells.

If speculation on the basis of the quinone-specificity of this pathway would suggest a quinone of the vitamin K type in this role, it raises the interesting possibility of regulation of TPNH levels by a factor of nutritional

importance. On the other hand, if a relation exists between the function of DT diaphorase, the biological activity of dicoumarol, and the nutrition requirement for vitamin K in the animal organism, then a much more specific role for DT diaphorase must be found.

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Discussion

PETERS: Were these citrate experiments done with mitochondria?

CONOVER: They were done with liver slices.

PETERS: I wasn't quite sure why you used citrate rather than isocitrate.

CONOVER: Well the primary reason was that the isocitrate we had was in the lactone form and we weren't quite sure whether this would be readily oxidized.

LOWENSTEIN: What concentration do you add?

CONOVER: Usually 10^{-3} M. Actually the experiments shown did not give the effect of TPN alone. It is not as high when added by itself as when added in the presence of vitamin K₃.

LOWENSTEIN: The other question I wanted to ask was: are the TPN diaphorase activities found in the cytoplasm and the mitochondria the same?

CONOVER: They are, as far as we have been able to tell. We have isolated the mitochondrial DT diaphorase and it exhibits identical properties with the cytoplasmic DT diaphorase.

LOWENSTEIN: What I was going to ask Dr. Ernster earlier was whether the TPN diaphorase from mitochondria is the same as transhydrogenases discussed by Dr. Kaplan?

ERNSTER: No.

LOWENSTEIN: What is the difference?

ERNSTER: That it doesn't transfer hydrogen between TPN and DPN. Kaplan's transhydrogenase is reported to be bound to the mitochondria (*J. biol. Chem.*, **205**, 1 (1953)) whereas about 95% of our enzyme is in the soluble cytoplasm (*Biochem. biophys. Res. Comm.*, **2**, 88 (1960)). Furthermore, DT diaphorase is strongly inhibited by dicoumarol.

LOWENSTEIN: Can you give a figure for the comparative rates of soluble TPN diaphorase and mitochondrial transhydrogenase?

ERNSTER: The activity of DT diaphorase ranges between 30 and 100 μ moles reduced pyridine nucleotide oxidized per min. and per g. rat liver (wet weight). I don't know what the activity of transhydrogenase is if you measure it with TPN and DPN and not with the analogues.

SINGER: I am wondering if you could refresh my memory on what compelling reason there is to believe that the transhydrogenase activity, as measured by Kaplan and others, can be ascribed to the action of the respiratory chain DPNH-dehydrogenase? I might add, to clarify my question, that while considerable transhydrogenase activity follows the respiratory chain DPNH-dehydrogenase throughout purification when the transhydrogenation of DPNH with DPN analogues is used as an assay, no DPNH-TPN transhydrogenation at all is shown by the purified enzyme. Thus the enzyme we have isolated is obviously not the one catalyzing transhydrogenations observed in mitochondria and its fragment involving TPN.

LOWENSTEIN: It has been purified by Kaufmann and Kaplan and has been found to remain intimately associated with the DPNH-electron transport system.

Mitochondrial Lipids and their Functions in the Respiratory Chain

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Mitochondria contain relatively large amounts of lipids and there is now a great deal of evidence which suggests that they play both structural and functional roles in mitochondrial metabolism. We have been studying the problem of lipid function in the respiratory enzyme system, both in intact phosphorylating mitochondria and in non-phosphorylating mitochondrial fragments.

Nature of mitochondrial lipids

Non-phosphorylating preparations of pig-heart muscle [1] were denatured with methanol and the lipid extracted with 40-60 light petroleum. The extract was then chromatographed on silicic acid (Mallinckrodt) and fractions eluted with increasing concentrations of diethyl ether in 40-60 light petroleum. Strongly adsorbing material at the top of the column was eluted with methanol. One result of such an analysis is

TABLE I
LIPID COMPOSITION OF PIG HEART-MUSCLE PREPARATION

Lipid	Concentration (mg. g. protein)	Percentage of total lipid
Total lipid	420	100.0
Phospholipid	378	90.1
Sterol	14.3	3.4
Neutral lipid	16.3	3.9
Ubiquinone	4.1	0.98

shown in Table I. The total lipid which amounts to 30% of the total dry weight of the preparation, contains 90% phospholipid while smaller amounts of sterol, neutral lipid and ubiquinone make up the total.

All the fractions were examined spectrophotometrically. Apart from ubiquinone which showed intense selective absorption at 275 m μ , a number

of fractions showed weak absorption at various wavelengths between 230 and 300 $m\mu$. There was, however, no evidence of spectra characteristic of the tocopherols or the vitamins K.

Extraction of lipids with organic solvents

An obvious way to tackle the problem of lipid function in mitochondrial particles is to remove the lipid by a suitable extraction procedure and observe the effect on enzyme activities. Nason and Lehman [2, 3] did this

TABLE II
EFFECT OF NUMBER OF EXTRACTIONS WITH LIGHT PETROLEUM ON THE SUCCINIC OXIDASE AND CYTOCHROME OXIDASE ACTIVITIES OF PIG HEART-MUSCLE PREPARATION

Number of extractions	Succinic oxidase ($\mu\text{l. O}_2/\text{hr.}/\text{mg. protein}$)			Cytochrome oxidase ($\mu\text{l. O}_2/\text{hr.}/\text{mg. protein}$)		
	Expt. No.			Expt. No.		
	1	2	3	1	2	3
0	259	320	242	810	1240	628
1	567	—	—	886	—	—
2	373	—	—	842	—	—
3	—	—	—	—	—	—
4	243	—	—	902	—	—
15	—	—	492	—	—	1056
20	—	960	—	—	—	—
30	—	—	540	—	1616	828
40	—	770	—	—	1880	—

Expt. No. 1: 2 ml. preparation (age 3 days) extracted successively with 4 ml. 40-60 light petroleum for 1 min.

Expt. No. 2: 1 ml. preparation (age 7 days) extracted successively with 1 ml. 40-60 light petroleum for 1 min.

Expt. No. 3: 1 ml. preparation (age 1 day) extracted successively with 5 ml. 40-60 light petroleum for 1.5 min.

Enzyme activities determined as described by Redfearn *et al.* [11].

simply by shaking the enzyme preparation with an organic solvent such as isooctane. After such a treatment it was found that the succinic- and DPNH-cytochrome *c* reductase activities had fallen considerably but that they could be restored to their original levels by adding α -tocopherol as a suspension in bovine serum albumin. Although it was later shown [4] that other substances would also reactivate solvent-extracted preparations a hypothesis was put forward implicating α -tocopherol as an essential component of the electron transport system [5]. The specificity of the reactivation by tocopherol was doubted by Deul *et al.* [6]. Redfearn and Pumphrey [7] then showed that the loss of enzymic activity after

shaking with an organic solvent was due principally to small amounts of the solvent retained in the enzyme suspension acting as a physical inhibitor. Removal of this residual solvent by physical means, e.g. dispersion with a surface-active agent, gave complete restoration of enzymic activities. These findings, which have since been confirmed by others [8, 9], make it necessary to be extremely cautious in evaluating the results of extraction-reativation experiments. Thus inactivation of an enzyme system due to an inhibition by the solvent must be clearly distinguished from inactivation brought about by the removal of lipid essential for some structural or functional role.

In a study of the effect of removal of lipid on enzyme activities, pig heart-muscle preparations were extracted with organic solvents by Nason's

TABLE III

EXTRACTION OF A PIG HEART-MUSCLE PREPARATION WITH DIETHYL ETHER

Number of extractions	Succinic oxidase (% original activity)	Succinic-cytochrome <i>c</i> reductase (% original activity)	Ubiquinone extracted (% total extractable ubiquinone)	Lipid extracted (% total lipid)
0	100	100	0	0
1	106	73	50	1
2	75	63	78	7
4	63	61	79	11
8	50	54	96	16

1 ml. preparation (35 mg. protein/ml.) extracted successively with 5 ml. peroxide-free diethyl ether for 1.5 min. Extracts washed with water, dried and solvent evaporated. Lipid residue weighed. Lipid dissolved in 40-60 light petroleum and ubiquinone separated and determined as described by Pumphrey and Redfearn [1]. Enzyme activity determined by the methods described by Redfearn *et al.* [11].

technique and the residual solvent removed by incubating the suspension in a Warburg manometer until solvent evolution had ceased. In this way, the effects of extraction could be studied without the additional complication of the inhibitory effects of the solvent itself. In experiments using 40-60 light petroleum as the solvent it was found that one or two extractions produced marked increases in the succinic oxidase and cytochrome oxidase activities (Table II). The amount of total lipid removed from the particles appeared to be small and less than 50% of the total extractable ubiquinone was removed even after forty successive extractions.

With diethyl ether, the endogenous ubiquinone of heart-muscle preparations could be extracted much more effectively. The results of an experiment are shown in Table III. 96% of the total extractable ubiquinone was removed after eight extractions with ether; the succinic oxidase and

succinic cytochrome *c* reductase activities had fallen to approximately 50% of the original activities. Polar solvents, such as ether and acetone, differ from non-polar solvents in that cytochrome oxidase is much more readily inactivated. Thus after one or two extractions with ether, cytochrome oxidase becomes the rate-limiting step in the respiratory chain.

Although much remains to be done on the correlation between enzymic activities and lipid content of respiratory chain particles, certain conclusions can be drawn from these preliminary experiments. Treatment of heart-muscle preparations with organic solvents appears to have three principal effects: (i) the physical action of the solvent producing changes in particle size and morphology, (ii) the removal of lipid from the particle by solution in the solvent, and (iii) the retention by the particles of small amounts of the solvent by surface adsorption or solution in the lipid. The first of these is undoubtedly the cause of the increased enzyme activities obtained after shaking the preparation with the solvent. This treatment probably results in the breaking down of large particles or aggregates into smaller particles or in changes in particle structure which allow an easier access of the reactants to the particles. The effect is probably analogous to the action of surface-active agents and the effect of freezing and thawing, processes which also result in increased enzyme activities. The solvent probably also displaces endogenous cytochrome *c*, which explains why solvent treated particles show a complete requirement for added cytochrome *c*.

With regard to the second effect, lipid is removed only with great difficulty by non-polar solvents but more readily by polar solvents, e.g. certain lipid components, such as ubiquinone, can be almost completely removed by extraction with ether. Cytochrome oxidase activity appears to be much more sensitive to polar solvents than to non-polar solvents.

The third effect, inhibition by the solvent itself, can be reversed by any one of a number of methods which depend on the removal or displacement of the solvent. It is interesting to note that this type of inhibition depends upon the structure of the particular solvent. Weber and Wiss [10] have shown with the *n*-alkanes, those with 6-7 carbon atoms are the most active. Even more potent inhibitors are the vitamin K₂ analogues with short side-chains. Weber and Wiss [10] showed that like the organic solvents, the inhibition due to these substances could be reversed by vitamin K₁, phytol and ubiquinone. Redfearn, Pumphrey and Fynn [11] suggested that the action of naphthoquinone inhibitors described by Ball, Anfinsen, and Cooper [12] could be explained in terms of a similar non-specific physical effect. Thus it could be imagined that the short lipophilic side-chains dissolve in the lipid phase of the particle with the projecting layer of large naphthoquinone nuclei acting as a barrier to the reactants. Recently, Herdlin and Cook [13] have presented evidence which appears to support this idea.

Ubiquinone (Coenzyme Q)

There is now a large amount of evidence which suggests that ubiquinone is a functional component of the respiratory chain. It is widely distributed in mitochondria, it undergoes enzymic oxidation-reduction and

TABLE IV
CONCENTRATION OF UBIQUINONE IN MITOCHONDRIAL PREPARATIONS

Preparation	Ubiquinone (μ moles g. protein)
Pig heart-muscle preparation	4.0
Horse heart-muscle preparation	4.0
Guinea-pig kidney mitochondria	1.6
Rat liver mitochondria	1.4
Pig kidney mitochondria	1.2
<i>Arum maculatum</i> spadix mitochondria	1.4

it is able to restore enzyme activity to solvent-extracted preparations [14, 15, 16].

A survey of ubiquinone concentrations in a number of tissue preparations has been made by the method described by Pumphrey and Redfearn [1] and some results are shown in Table IV. The concentration of

TABLE V
RELATIVE CONCENTRATIONS OF THE CYTOCHROMES AND UBIQUINONES

Preparation	Concentration (μ moles g. protein)					Ratio				
	<i>a</i>	<i>b</i>	<i>c</i>	<i>c</i> ₁	UQ	<i>a</i>	<i>b</i>	<i>c</i>	<i>c</i> ₁	UQ
Pig heart-muscle preparation	0.74	0.6	0.085	0.37	4.39	2.0	1.6	0.23	1.0	12.0
Rat liver mitochondria	0.13	0.13	0.12	0.14	1.41	0.93	0.93	0.9	1.0	10.0

The concentrations of cytochromes *a*, *b*, and *c* were determined using the wavelengths and molar extinction coefficients given by Chance and Williams [26] and *c*₁ from the data of Green *et al.* [27].

ubiquinone relative to the cytochromes has also been determined in pig heart-muscle preparations and rat liver mitochondria (Table V). The cytochrome concentrations were determined spectrophotometrically after solubilization of the preparations in sodium cholate. It will be seen that in

both cases, on a molar basis, ubiquinone is present in a considerable excess over the cytochromes. On an electron-carrying basis the ubiquinone/cytochrome ratio is, of course, increased further by a factor of two to give ratios of 20–24. The reason for this large excess of ubiquinone is not clear at the moment although it has important consequences when discussing its possible function in the respiratory chain. It is interesting to note the extraction experiments already described indicate that relatively large amounts of ubiquinone may be removed from mitochondrial particles without apparently having drastic effects on enzymic activities. Thus it is possible that only the stoichiometric amount is necessary for efficient operation of the respiratory chain.

The results of a study of the kinetics of ubiquinone reactions in heart-muscle preparations, the action of inhibitors on these reactions and a discussion of the possible function of ubiquinone in the non-phosphorylating respiratory chain have been presented recently [17, 15, 16]. To summarize briefly, the rate of reduction of ubiquinone by DPNH or succinate is less than the total electron flux as measured by the substrate oxidase rates; most of the endogenous ubiquinone appeared to be accessible to both substrates; inhibitor studies indicate that its site of action is between the flavoproteins and the antimycin-A-sensitive region. Three possible schemes for the position of ubiquinone in the non-phosphorylating chain can be put forward [16]. These are: (i) that ubiquinone is on the main respiratory chain mediating the reaction between the flavoproteins and the cytochromes. (ii) that it reacts only with the flavoproteins to form a blind-alley pathway, and (iii) that it is on a branch pathway linking the flavoproteins with cytochrome c_1 via the antimycin-A-sensitive region.

In order to try to elucidate the mode of action of ubiquinone in the intact phosphorylating system we have begun experiments with rat-liver mitochondria. The mitochondria were prepared by a modification of the method of Schneider and Hogeboom [18] and ubiquinone determined by the method of Pumphrey and Redfearn [1]. Respiratory control and P/O ratios were determined with the oxygen electrode [19], and steady-state oxidation-reduction levels of ubiquinone were measured in the different metabolic states [20] of the mitochondria. Typical spectra are shown in Fig. 1. It can be seen that in the absence of added substrate or ADP (State 1) the ubiquinone is 45% reduced while on adding ADP (State 2) it becomes 38% reduced. In the presence of added substrate (succinate) but no ADP (State 4) the ubiquinone is 80% reduced but on adding ADP (State 3) falls to 72% reduction. The results of experiments on four different mitochondrial preparations are shown in Table VI. When succinate is the substrate ubiquinone is largely reduced (80–89%) in State 4 and becomes less reduced (70–86%) in State 3 while the corresponding figures for β -hydroxybutyrate are 53–72% (State 4) and 40–63% (State 3).

Experiments with antimycin A, which inhibits oxidation of ubiquinol, have shown that the enzymically reducible ubiquinone is only 80-90% of the total. Thus succinate in State 4 is actually giving complete reduction

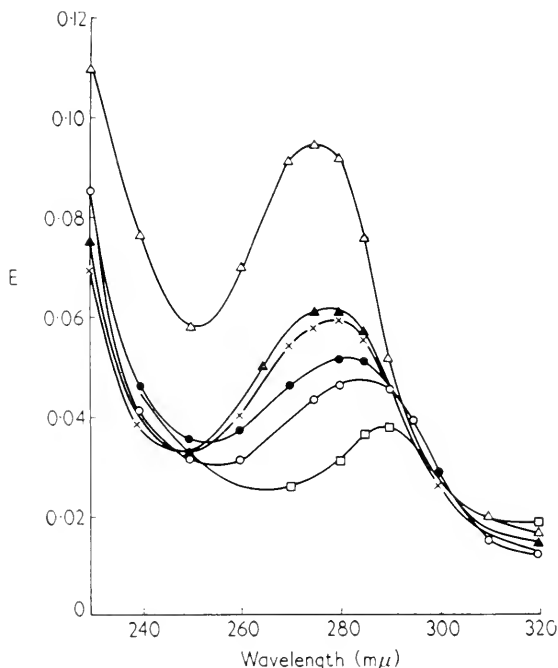


FIG. 1. Steady-state oxidation-reduction levels of ubiquinone in rat-liver mitochondria.

- total ubiquinone (oxidized);
- total ubiquinone reduced with NaBH_4 ;
- ×— P_i + air; —▲— P_i + ADP + air;
- P_i + succinate + air; —●— P_i + succinate + ADP + air.

of the enzymically active material, while β -hydroxybutyrate in State 4 is giving about 80% reduction.

These changes are qualitatively similar to those reported by Chance [21] for guinea-pig kidney mitochondria and support the view that the oxidation of ubiquinol is blocked in the absence of a phosphate acceptor by an inhibitory interaction which could involve energy conservation as a high-energy intermediate $\text{UQH}_2 \sim \text{I}$ by a series of reactions as follows:

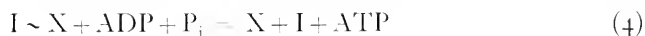
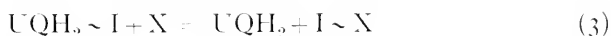
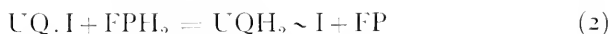


TABLE VI

STEADY-STATE OXIDATION-REDUCTION LEVELS OF UBIQUINONE IN VARIOUS METABOLIC STATES OF RAT-LIVER MITOCHONDRIA

Preparation number	Ubiquinone concentration (μ moles/g. protein)	Substrate	Steady-state percentage reduction of total ubiquinone			
			+P _i + ADP+O ₂	+P _i +O ₂	+ADP+ O ₂	+O ₂
14	1.3	endogenous	38	45	44	—
		succinate	72	80	80	82
15	1.6	endogenous	45	—	—	—
		β -hydroxy- butyrate	44	64	68	—
		succinate	80	85	87	88
17	1.3	endogenous	33	—	—	—
		β -hydroxy butyrate	30	53	—	—
		succinate	69	81	84	—
18	2.2	endogenous	63	—	—	—
		β -hydroxy- butyrate	63	72	72	—
		succinate	86	89	88	—

Steady-state determinations were made with a reaction mixture of the following final composition: Sucrose, 107 mM; MgCl₂, 15.5 mM; KCl, 25 mM; Na₂HPO₄-KH₂PO₄, pH 7.4, 12.5 mM (or tris-HCl, pH 7.4, 18.8 mM); ADP, 18 mM; sodium β -hydroxybutyrate, 4.5 mM; sodium succinate 3.0 mM; mitochondrial protein, approx. 6 mg. ml. Total volume 1.4 ml. Mixture aerated for 30 sec. Temp. 17-20.

Hatefi [22] has also described results of experiments on beef-heart mitochondria which support such a role for ubiquinone. He found also that when phosphate in the medium was replaced by tris the ubiquinone went into the completely oxidized state. This was interpreted as being due to the release of the inhibitory effect of phosphate on the oxidation of ubiquinol. In the present work this phosphate effect could not be demonstrated in rat-liver mitochondria; the steady-state levels of ubiquinone were almost the same in the absence of phosphate, in the presence of phosphate, and in the presence of ADP without added phosphate (Table VI).

Recently a number of workers have put forward hypotheses implicating phosphorylated derivatives of quinones in oxidative phosphorylation [23, 24, 25]. Ubiquinol monophosphate, a possible intermediate in these postulated reaction mechanisms has been synthesized by Dr. K. J. M. Andrews of Roche Products Ltd., Welwyn. In a preliminary experiment this substance was added to rat-liver mitochondria in the presence of ADP but no stimulating effect on the rate of oxygen uptake was observed. Also

spectrophotometric examination of light petroleum extracts of mitochondria did not reveal anything with the spectral characteristics of ubiquinol monophosphate.

Summary

1. Mitochondrial preparations contain relatively large amounts of phospholipids with smaller concentrations of neutral lipid, sterol and ubiquinone.

2. The extraction of mitochondrial preparations with organic solvents was studied and three principal effects were distinguished.

3. The concentrations of ubiquinone in a number of mitochondrial preparations were measured. Ubiquinone concentrations with respect to the individual cytochromes were shown to be relatively high.

4. The steady-state oxidation-reduction levels of ubiquinone in rat liver mitochondria in various metabolic states have been measured. The possible role of ubiquinone in oxidative phosphorylation was discussed.

Acknowledgment

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Discussion

ZIEGLER: I would like to point out that it would be difficult to measure the initial rates of endogenous Q reduction by the method Dr. Redfearn used. The particles contain a large excess of Q relative to the cytochromes or flavoprotein and only part of it may be rapidly reduced in a blocked system. In order to measure initial rates at 22° the reaction would have to be stopped in less than a second and I believe your reaction times were of the order of several seconds. Later in this Symposium we will present data which show that the rate of reduction of exogenous Q is fully compatible with the assumption that it functions as an electron carrier between the flavoprotein and cytochrome *c*. The turnover of the flavoprotein with Q as the acceptor is more rapid than it is in the intact particle with oxygen as the acceptor.

REDFEARN: This is a derivative particle that you are using?

ZIEGLER: The naturally occurring quinone, Q₁₀, is reduced as rapidly as the synthetic Q homologues.

REDFEARN: We have measured the rate of reduction of exogenous ubiquinone in our preparation, and found the rate to be very much slower than that of the reduction of the endogenous material.

ZIEGLER: You do require lipids. Coenzyme Q₁₀ is extremely insoluble in water, and by adding a mixture of phospholipids you can increase its effective concentration to the point where you can use it as the final electron acceptor.

REDFEARN: I would like to add that in experiments we did with Dr. Chance we measured the rate of reduction of endogenous ubiquinone in the double beam spectrophotometer at the same time as we measured the rate by the extraction procedure, and we got very close agreement.

CHANCE: We have been interested in the maximal rate at which the endogenous Q could be reduced. I think that Dr. Redfearn and I had already observed rates at 22° of about 5 micromoles per hour per milligram protein for the reduction of endogenous Q on adding succinate to the CN-inhibited system. By using a rapid flow apparatus and more active preparations we have observed values of Q-reductase activity up to this level, which is a rather high activity, but this is still somewhat less than the rates of oxygen reduction.

SINGER: I was wondering, since you did not commit yourself, which of the three possible hypotheses of the mode of action you favour, and whether the sum total of the data presented today plus those you published in the *Biochemical Journal* do not point to a possible function of Q in interchain electron transport?

In weighing the evidence it is well to remember that what matters is not that under certain sets of conditions the rate of cycling of ubiquinone approaches that of the respiratory chain, but rather that it is relatively easy to produce conditions under which the turnover of ubiquinone is lower than the rate of succinate oxidation. The latter type of experiment would not suggest that ubiquinone is an obligatory component of the electron transport chain, but it would by no means exclude its function as an interchain lipid. This function would, of course, also lead to a reduction of cytochromes c and c_1 but not necessarily in the same chain.

REDFEARN: I, of course, rather favour this idea and I discussed it at some length in a recent paper, but I didn't want to commit myself here. I think this possibility fits the results well but we can't exclude the other possibilities.

ESTABROOK: I have a question of terminology on your very interesting observation. You lose only 20% of the succinate oxidase activity on removing 96% of what you said was extractable Q_{10} . Is this total ubiquinone or that extracted by your solvent system? Was there still over 10% remaining?

REDFEARN: Yes.

WILLIAMS: I wanted to ask whether it would be a logical consequence of Dr. Singer's hypothesis, that you should be able to isolate chains which do not contain ubiquinone?

REDFEARN: Yes, that is what we are trying to do. We find it very difficult to remove the remaining few per cent of ubiquinone. As you continue extracting you remove more and more phospholipids and other structural lipids and then you start losing activity. It is very difficult to remove 100% of the ubiquinone without removing other lipids.

The Functional Link of Succinic Dehydrogenase with the Terminal Respiratory Chain

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It is our purpose to examine some energetic aspects of the electron transport system catalyzing the aerobic oxidation of succinate in intact liver mitochondria. It has been generally accepted that the oxidation of succinate is completely independent of electron transport and phosphorylation in the DPN-flavin region of the respiratory chain. Support for this concept came from the findings that mitochondria either depleted of DPN, or in the presence of amytal, as well as non-phosphorylating submitochondrial preparations are fully capable of catalyzing the aerobic oxidation of succinate. Therefore it seemed likely that cytochrome *b* was the site of entrance for the electrons coming from succinic dehydrogenase; the two phosphorylations in the cytochrome region of the respiratory chain could then account for the net phosphate uptake occurring during succinate oxidation.

Renewed interest in this question has emerged subsequent to the recent work of Chance and Hollunger [1, 2], and of Klingenberg *et al.* [3, 4], who found that the extent of reduction of mitochondrial pyridine nucleotide is greatly increased by the addition of a flavosubstrate, succinate, or glycerol 1-phosphate.

A different approach to the question of the interaction between succinic dehydrogenase and the DPN-flavin region of the respiratory chain recently has been possible because of the finding [5, 6] that intact liver mitochondria, when depleted of high energy phosphate, are no longer capable of oxidizing succinate at any appreciable rate unless ATP is added, or synthesized in the system. Furthermore, the beneficial effect of ATP is not abolished in the presence of uncoupling agents.

The depletion of mitochondrial high energy phosphate and the inhibition of succinate oxidation

An experiment illustrating the depression of the capacity for succinate oxidation in rat liver mitochondria is shown in Fig. 1. Addition of arsenate

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(Expt. (a)) to liver mitochondria in the presence of succinate elicited a respiration which was less than half of that obtained in the presence of either dicoumarol (Expt. (a)), or phosphate and a phosphate acceptor. The increased respiration elicited by arsenate was due to a partial release of respiratory control. Subsequent addition of dicoumarol released the respiratory control completely. When the mitochondria were preincubated (Expt. (b)) with arsenate for 3 min. prior to the addition of succinate the respiration was about half maximal, but even this level was reached only after a lag phase. If on the other hand dicoumarol was added during the preincubation (Expt. (c)) together with arsenate, the rate of succinate oxidation remained low upon prolonged incubation (about 10% of the maximum).

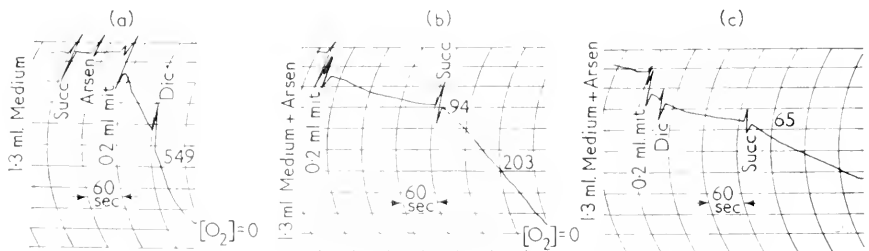


FIG. 1. Inhibition of succinate oxidation in rat liver mitochondria incubated with arsenate and dicoumarol [6]. Concentrations of the reagents in a final volume of 1.5 ml. were as follows: 0.05 M KCl, 0.033 M tris buffer pH 7.5, 0.008 M MgCl₂, 0.05 M sucrose, 0.013 M succinate (Succ.), 0.002 M arsenate (Arsen.), 0.00006 M dicoumarol (Dic.). Mitochondria from 400 mg. rat liver wet weight (about 8 mg. protein). The substances were added at the points indicated. "1.3 Medium" stands for KCl, tris, MgCl and sucrose added in a volume of 1.3 ml. Oxidation rate of succinate is given in μ atoms oxygen per min.

A possible interpretation of these findings was suggested by experiments in which arsenate was found to deplete the mitochondria of their endogenous phosphate. When mitochondria labelled with ³²P were incubated in the presence of arsenate (Table I) an almost complete depletion of mitochondrial phosphate took place within a few minutes. Addition of succinate largely prevented this effect of arsenate. The prevention was ascribed to a reincorporation of inorganic phosphate into ATP by way of aerobic phosphorylation, since the oxidation of succinate is only partly uncoupled by arsenate. Addition of respiratory inhibitors such as antimycin A or KCN abolished the succinate effect on the arsenate-induced depletion.

Thus the initial low rate of succinate oxidation after preincubation with arsenate was considered to be a consequence of the loss of high energy phosphate from the mitochondria, and the gradual increase in the rate of

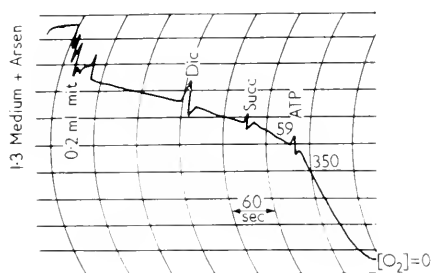


FIG. 2. Stimulation of succinate oxidation by ATP in rat liver mitochondria preincubated with arsenate and dicoumarol [5]. Experimental conditions as in Fig. 1. 0.001 M ATP (ATP) was added at the point indicated.

oxidation, as a consequence of the resynthesis of high energy intermediates taking place during the oxidation of succinate. When this resynthesis was abolished by the presence of an uncoupling agent, the depression of succinate oxidation became permanent. Under these conditions, added ATP was required for stimulating the oxidation of succinate (Fig. 2). No stimulation of the oxidation rate was observed when ATP was replaced by AMP or EDTA.

TABLE I

DEPLETION OF MITOCHONDRIAL ENDOGENOUS PHOSPHATE BY ARSENATE AND PROTECTION BY SUCCINATE

^{32}P -labelled mitochondria from 500 mg. rat liver (wet weight) were incubated in open tubes at 30° . After 7 min., 1 ml. of the incubation mixture was filtered through a Celite layer as reported elsewhere [12]. Each tube contained in a final volume of 5 ml.: 0.05 M KCl, 0.03 M tris buffer pH 7.5, 0.125 M sucrose, and when indicated 0.01 M succinate, 0.003 M arsenate, 2 μg . antimycin A, 0.001 M KCN. As in Table II and III, the number of counts is here indicative of the amounts of endogenous phosphate which remained in the mitochondria after preincubation.

Additions	Counts min. ($\times 10^{-2}$)
None	700
Arsenate	100 (14.4%)
Succinate	521 (73.5%)
Arsenate + succinate	421 (60.2%)
Arsenate + succinate + Antimycin A	223 (31.9%)
Arsenate + succinate + KCN	128 (18.3%)

The concept that intact mitochondria have a strict requirement for high energy phosphate and not merely of inorganic phosphate in order to maintain a high rate of succinate oxidation is supported further by the following findings.

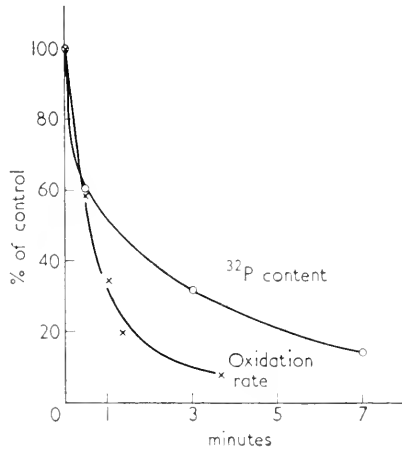


FIG. 3. Correlation of the inhibition of succinate oxidation with the depletion of mitochondrial phosphate. Mitochondrial endogenous phosphate measured as described in Table I.

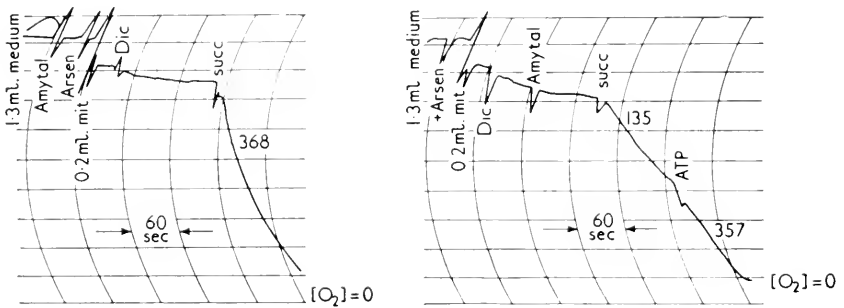


FIG. 4. Prevention of the inhibition of succinate oxidation by amytal [6]. Experimental conditions as in Fig. 1. 0.002 M amytal was added at the point indicated.

The presence of a five-fold molar excess of inorganic phosphate during the preincubation of the mitochondria with arsenate completely prevented the inhibition of succinoxidase activity. On the other hand, if the same concentration of inorganic phosphate was added to the mitochondria *after*

they had been depleted of high energy phosphate by the arsenate-dicoumarol pretreatment, no stimulation of the oxidation rate was observed, and added ATP was required in order to restore the succinoxidase activity.

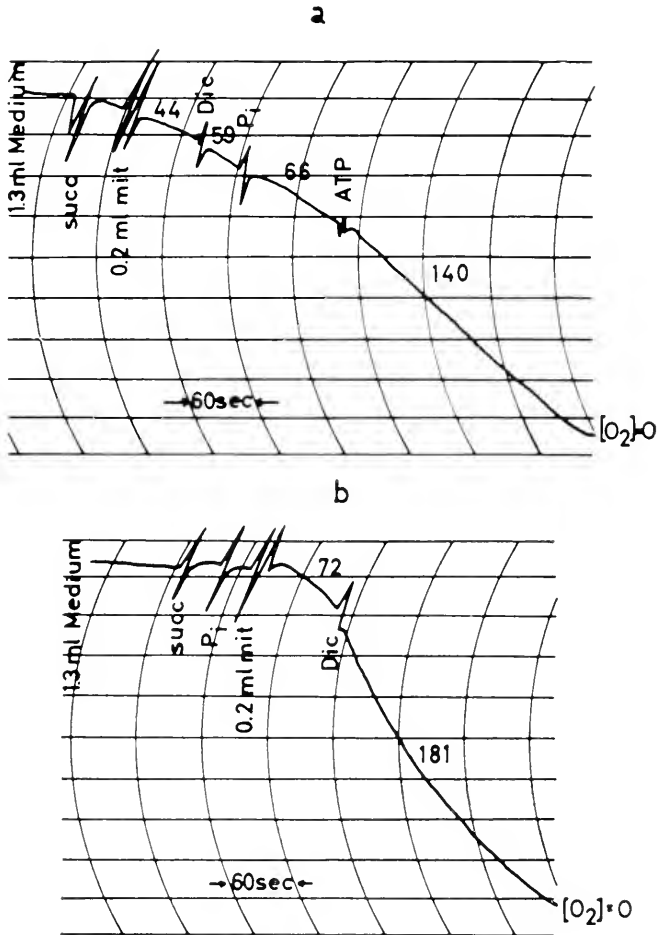


FIG. 5. Stimulation of succinate oxidation by inorganic phosphate and ATP in pretreated rat liver mitochondria [6]. Experimental conditions as in Fig. 1. The mitochondria used in this experiment were pretreated for 5 min. at 30° with 0.0001 M DNP plus 0.001 M AMP, and then washed with 0.25 M sucrose.

As shown in Fig. 3, the time of preincubation with arsenate necessary for inhibiting succinate oxidation corresponded approximately to the time required for depleting the mitochondria of high energy phosphate.

Additional support for the above conclusion was obtained with the use of amytal. As reported elsewhere, the depleting effect of arsenate on the

mitochondrial high energy phosphate was almost completely abolished in the presence of 2 mM amytal. In agreement with this experiment, it was found (Fig. 4) that when amytal was added prior to, or together with, arsenate a high respiration ensued upon the addition of succinate. On the other hand, if amytal was added *after* the preincubation with arsenate, the succinoxidase activity was greatly inhibited and again, added ATP was required to increase the oxidation rate.

It could be demonstrated clearly that the depression of the capacity for succinate oxidation was caused by the depletion of mitochondrial high energy phosphate compounds, and *not* by the presence of arsenate itself. This was accomplished by the use of 2,4-dinitrophenol (DNP) plus AMP to pretreat mitochondria which were then washed free of the depleting agents. The succinoxidase activity of these preparations was very low and could be stimulated more than two-fold by the addition of ATP (Fig. 5 (a)). Inorganic phosphate also could increase the oxidation rate but only if added before the uncoupling agent and together with succinate (Fig. 5 (b)). Under these conditions high energy phosphate compounds could be synthesized by the mitochondria.

The question of oxaloacetate

Oxaloacetate is known to be a competitive inhibitor of succinic dehydrogenase, and its accumulation has been considered as chiefly responsible for the inhibitions of succinate oxidation observed by different workers. Furthermore, a protective effect of ATP against the inhibition induced by oxaloacetate has been reported by Pardee and Potter [8] and by Tyler [9]. Therefore it was necessary to examine in greater detail the mechanism by which energy is provided for the activation of succinate oxidation, and also the possible ways by which oxaloacetate may interfere with the mitochondrial oxidation of succinate.

The possibility that the accumulation of oxaloacetate, *per se*, could be responsible for the low rate of succinate oxidation after the arsenate-dicoumarol preincubation, has been excluded by three types of experiments:

(a) The inhibition was not relieved by the addition of cysteine sulphinate, in the presence of amytal. Control experiments showed that cysteine sulphinate did remove an inhibition of succinate oxidation due to *added* oxaloacetate in agreement with the finding of Singer and Kearney [10] who have demonstrated that cysteine sulphinate transaminates oxaloacetate to aspartate.

(b) It would be anticipated that ATP could be replaced by GTP (or ITP) if ATP was acting by removing oxaloacetate via the oxaloacetic carboxylase reaction since this reaction specifically utilizes GTP (or ITP). Under our conditions little stimulation of succinate oxidation was observed

after the addition of GTP or ITP as compared with that induced by ATP. Furthermore, only trace amounts (less than 5×10^{-7} M) of phosphoenolpyruvate, the product of the oxaloacetic carboxylase reaction, could be detected after the addition of ATP.

(c) No measurable amounts of oxaloacetate could be detected after the arsenate-dicoumarol preincubation; the assay was sensitive to concentrations of oxaloacetate in the incubation mixture as low as 5×10^{-8} M [11].

Although these findings seem to preclude a possible involvement of oxaloacetic acid as directly responsible for the observed inhibition of succinate oxidation, some indication was obtained that the presence of oxaloacetate was necessary during the preincubation in order to obtain the inhibited state. Addition of cysteine sulphinate during the arsenate-dicoumarol preincubation, which could be expected to remove all the oxaloacetate formed from endogenous substrates, resulted in a complete protection of succinate oxidation.

The substrate level phosphorylation compartment and the energy source for the activation of succinate oxidation

Evidence has been presented that the α -ketoglutarate-linked substrate-level phosphorylation can give rise to an ATP which is not directly available to the DNP-induced ATPase [12]. From this finding the concept was developed that the ATP originating from the substrate level phosphorylation is compartmentalized in the mitochondria and that accessory reac-

TABLE II

EFFECT OF AMP AND OF VARIOUS SUBSTRATES ON THE DNP-INDUCED DEPLETION OF MITOCHONDRIAL ENDOGENOUS PHOSPHATE [12]

Each tube contained in a final volume of 3 ml.: 0.001 M AMP, 0.0001 M DNP, 0.003 M glutamate, 0.003 M β -hydroxybutyrate, 0.003 M oxaloacetate and 0.003 M succinate; other experimental conditions as in Table I; 0.001 M $MgCl_2$ was also added in Expt. 2. Time of incubation 7 min. in Expt. 1 and 5 min. in Expt. 2.

	Additions	Counts/sec.
Expt. 1	none	429
	DNP	277 (65.0%)
	DNP + succinate	170 (39.8%)
	DNP + AMP	114 (26.7%)
Expt. 2	none	326
	DNP	238 (73.2%)
	DNP + Glutamate	212 (65.2%)
	DNP + β -hydroxybutyrate	223 (68.6%)
	DNP + oxaloacetate	134 (41.1%)
	DNP + succinate	130 (40.1%)

tions are required for transferring phosphate from this ATP to external ADP. Indications were obtained for the following mechanisms being operative in this transfer of phosphate: a double adenylate kinase, the oxaloacetic carboxylase-pyruvic kinase and the activation of succinate oxidation discussed in the present paper. An experiment showing the effectiveness of these three mechanisms in transferring phosphate in order to render ATP available to the DNP-induced ATPase is illustrated in Table II. When ^{32}P -labelled mitochondria were incubated in the presence

TABLE III

EFFECT OF CYSTEINE SULPHINATE AND OXALOACETATE ON THE RELEASE OF ^{32}P FROM MITOCHONDRIA DURING INCUBATION WITH ARSENATE [7]

Each tube contained in a final volume of 3 ml.: 0.05 M KCl, 0.03 M tris buffer pH 7.5, 0.125 M sucrose, 0.01 M MgCl_2 and, when indicated, 0.003 M arsenate, 0.005 M cysteine sulphinate, 0.002 M amytal, 0.003 M oxaloacetate. " ^{32}P -labelled mitochondria" from 500 mg. liver. Time of incubation, 5 min. Temperature, 30°.

Addition	Counts/sec.	
	Expt. 1	Expt. 2
None	482	525
Arsenate	76	73
Arsenate, cysteine sulphinate	—	211
Amytal	429	—
Amytal, oxaloacetate	203	—
Amytal, oxaloacetate, cysteine sulphinate	404	—
Arsenate, amytal	339	—
Arsenate, amytal, oxaloacetate	117	—

of DNP a partial release (about one-third) of the ^{32}P took place. Addition of AMP, succinate or oxaloacetate enhanced the releasing effect of DNP. No such effect was obtained with β -hydroxybutyrate or glutamate, indicating that the release induced by succinate or oxaloacetate was not due in an unspecific manner to the presence of an oxidizable metabolite.

The capacity of oxaloacetate in removing ^{32}P from the mitochondria is also illustrated in Table III. When the depleting effect of arsenate was removed by the presence of amytal (see Fig. 4), addition of oxaloacetate induced again a large release of ^{32}P from the mitochondria (Expt. 1). Oxaloacetate was able, alone or in the presence of amytal, to remove ^{32}P from the mitochondria, and the depleting effect of oxaloacetate was removed by the addition of cysteine sulphinate (Expt. 1). A partial but significant protection against the arsenate-induced release of ^{32}P from labelled mitochondria was also obtained when cysteine sulphinate was added to the incubation medium (Expt. 2). Thus the capacity of cysteine

sulphinate to prevent the inhibition of succinate oxidation when added to the mitochondria during the incubation with arsenate and dicoumarol, can be explained on the basis of the ability of cysteine sulphinate to maintain, by rendering the oxaloacetic carboxylase reaction inactive, high energy compounds in the substrate level phosphorylation compartment of the mitochondria.

On the basis of the present findings it appears that oxaloacetate besides its known function as competitive inhibitor of succinic dehydrogenase, possesses also the capacity of competing, through the oxaloacetic carboxylase reaction, in the utilization of the high energy phosphate compounds required for succinate oxidation. This additional property of oxaloacetate appears to be of particular significance in conditions where the generation of high energy phosphate compounds from respiratory chain phosphorylations is blocked by the addition of uncoupling agents. In these conditions the oxidation of succinate, which otherwise can dispose of the high energy intermediates formed in the last two respiratory chain phosphorylations, will be dependent, as sole source of energy, on the ATP which originates from the α -ketoglutarate-linked substrate-level phosphorylation.

The activation of succinate oxidation

The experimental evidence reported above supports the concept that in intact mitochondria the aerobic oxidation of succinate must proceed through a thermodynamically unfavourable reaction which requires the investment of energy. This energy-demanding reaction appears to be characteristic only of phosphorylating mitochondrial preparations, since no such requirement has been demonstrated for non-phosphorylating succinoxidase preparations.

Although no conclusive evidence exists at present regarding the nature of the high energy intermediate formed, it seems conceivable that this intermediate must possess the following properties: (a) it must be different from the two high energy intermediates which provide the two *net* phosphorylations occurring during the aerobic oxidation of succinate; (b) the energy of this additional intermediate can be used directly or indirectly for the reduction of the mitochondrial pyridine nucleotide; this conclusion is supported by the findings made in collaboration with Klingenberg [11] that the stimulation of succinate oxidation by ATP in arsenate-dicoumarol pretreated mitochondria is paralleled by a reduction of mitochondrial pyridine nucleotide; (c) the formation of this high energy intermediate is not impaired by the presence of uncoupling agents. This finding suggests that the energy required for activation of succinate oxidation and for reduction of pyridine nucleotide cannot be supplied by a non-phosphorylated intermediate of the $X \sim I$ type, because the latter has the

property of being hydrolyzed by DNP. On the other hand, this result is consistent with those hypotheses in which a reduced phosphorylated electron carrier of the $XH \sim P$ type is formed at the expense of ATP in the presence of uncoupling agents.

Therefore, it appears likely that once the reduced phosphorylated intermediate is formed, it can either transfer electrons to the cytochrome system by which ATP is regenerated, or to DPN in which case reduced pyridine nucleotide is formed and inorganic phosphate is liberated. Further work is in progress in order to define more precisely the reactions by which the oxidation of succinate by the terminal respiratory chain requires a supply of high energy phosphate.

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Discussion

CHANCE: I think Dr. Azzone's paper has a great deal of information in it, it takes time to digest. His remark about the non-phosphorylating preparation oxidizing succinate directly is surely one that we must not forget in postulating mandatory succinate oxidation, and I guess that it is one thing that just isn't explained yet by your mechanism. We have the feeling that the ATP requirement for succinate oxidation and ATP requirement for reduction of pyridine nucleotide may not be identical in detail although they appear to be identical in the kind of experiments that you have been doing. I think that the amytal sensitivity that Dr. Ernster has already referred to gives a hint that this process does involve carriers of the respiratory chain. I don't quite understand the basis on which you conclude the uncoupling agents don't impair the formation of DPNH, or maybe you meant they didn't impair the reactivation of succinate oxidation, because I believe it is clear from our experiments that the DPNH formation is highly sensitive to uncoupling agents.

AZZONE: Well, I think we must make a distinction here. If you mean that in State 3, DPNH gets easily oxidized, then I agree with you that addition of dinitrophenol, which induces a State 3 condition, also makes it more difficult to observe DPN reduction. But our conclusion that addition of uncoupling agents does not

inhibit DPN reduction, was derived from the finding (reported by Dr. Ernster) that the energy of ATP could be used for reducing DPN in the presence of dinitrophenol.

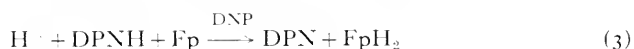
According to the phosphoryl-flavin theory [L6w, Siekevitz, Ernster and Lindberg, *Biochim. biophys. Acta* **29**, 392 (1958)] ATP can react with the diaphorase flavin giving rise to a reduced phosphorylated electron carrier:



It has been suggested that Reaction (1) is not sensitive to the uncoupling agents, and therefore the energy of ATP can be used, through the intermediate $\text{FpH} \sim \text{P}$, for reducing DPN (Reaction (2)):



In the presence of dinitrophenol Reaction (2) will take place from right to left without the activation of inorganic phosphate (Reaction (3)):



Thus the sum of Reactions (1), (2) and (3) will account for an ATP-ase activity.

CHANCE: I still think there is a discrepancy because it is the rate of reduction of DPN (the second equation) that we observed optically to be inhibited.

AZZONE: The fact that the rate of reduction of DPN is lower in the presence than in the absence of dinitrophenol can be explained on kinetic reasoning.

Once the high energy intermediate postulated in our hypothesis has been formed during succinate oxidation [Azzone, Ernster and Klingenberg, *Nature, Lond.* **188**, 552 (1960)] it can be either utilized for reducing DPN, or reoxidized by the cytochrome system. The higher the electron flow toward the oxygen the lower will be the utilization of the intermediate in the backward reaction for reducing DPN.

ERNSTER: We do accept the fact that the level and rate of DPN-reduction may be low in the presence of an uncoupling agent which allows full respiration. However, the point we wish to stress is this: Is it at all possible to obtain an ATP-induced DPN-reduction (no matter how little) in the presence of a fully uncoupling concentration of dinitrophenol or dicoumarol? I think our data clearly show that it is.

HOLTON: Before the experiments of Chance and Hollunger were published, when one got an activation of oxidation of succinate in mitochondria one normally regarded this as evidence that the mitochondria were breaking up. One knows very well that in intact mitochondria succinate oxidation is rather slow while in mitochondrial fragments it is extremely rapid, so it is clear that the structural state of the mitochondrion can have some influence on the rate at which it oxidizes succinate quite apart from mechanisms of the type postulated by Chance and Hollunger. I wonder whether it might be as well to keep in mind that changes in the rate of oxidation of succinate can be mediated by changes in the structural state of the mitochondrion, that changes in structural state can be brought about by changes in the ATP:ADP ratio and the possibility that these mechanisms of succinate oxidation involving energy requirement and oxidation via DPNH are not the only way of explaining an acceleration of succinate oxidation under any particular experimental conditions.

AZZONE: We have not yet conclusive evidence about the chemical reaction requiring the investment of energy. However, even if it is assumed that the ATP stimulation is the consequence of a structural effect we still have to account for the formation of a high energy intermediate, controlling the oxidation of succinate which precedes the mechano-chemical utilization of ATP.

HOLTON: We must consider the availability of the succinate to the succinic hydrogenase besides the possibility that its oxidation requires an investment of energy.

AZZONE: According to the mechanism we have proposed, in non-phosphorylating preparations it is possible for the electron derived from succinate to proceed through a sequence of electron carriers each of which possesses a higher redox potential than the preceding one; on the contrary, in intact mitochondria there is a thermodynamically unfavourable step which must be circumvented by the investment of energy.

WILLIAMS: I should like to say that, as Dr. Azzone is by now aware, we have found, (a) that cysteine sulphinic acid reactivates rat liver mitochondria under conditions as similar as we could get to the ones described in his paper in *Nature*, (b) that, although pyruvate affords some degree of protection, α -ketoglutarate does not, although it does maintain its substrate level phosphorylation. However, I think it may be better to emphasize the measure of our agreement and to suggest that you are now coming very close to saying that oxaloacetate is necessary. I think our disagreement is then not so great and we can leave open the details of how oxaloacetate exerts this inhibition and how ATP relieves this inhibition. In Tyler's work ATP relieved the inhibition without altering the oxaloacetate concentration, so looking for PEP may not help us, and here I had wondered, as had Dr. Holton, whether the ATP acts by segregating the oxaloacetate from the succinic dehydrogenase, although there are no major optical density changes during this process.

SLATER: I should like to support what Dr. Williams said a moment ago, that a very important point to look at is how ATP reverses oxaloacetate inhibition and I should like to bring over a suggestion of my colleague Dr. Hülsmann, that you look into the possibility that ATP is activating oxaloacetate removal by reacting with endogenous substrate and forming acetyl-coenzyme A which promotes the removal of oxaloacetate; this is based on some recent experiments and explains quite a lot of phenomena including the Amytal experiments, in the presence of which oxaloacetate is not formed.

AZZONE: We have not been able to demonstrate the presence of oxaloacetate in the arsenate-dicoumarol preincubated mitochondria so one must postulate that oxaloacetate is compartmentalized in such a way that it cannot be reached by chemical analysis.

MITCHELL: I should like to raise a point that may bring together Dr. Slater's views and those of Dr. Holton. The suggestion that I am going to make comes from work on micro-organisms which Dr. Moyle and I have been doing recently. It so happens that in micrococci you can show that the oxidation of succinate can be blocked by DNP when the membrane is intact. It can also be blocked by mercury compounds or by arsenite. But, it can be demonstrated that the blocking of oxidation by these inhibitors is not a direct effect on the oxidation system but is due to blockage of the system by which "succinate" passes into the cell. If you

give a short period to allow the "succinate" to enter the cell before adding the inhibitor, the succinate that disappeared from the medium before the inhibitor was added subsequently becomes oxidized in the cell at almost the normal rate. If, however, you break the membrane before adding these inhibitors of "succinate" transport, succinate oxidation is not inhibited. There is not time to go into details, but we have good reasons for believing that succinate goes through the membrane either as succinyl-CoA or as succinyl-lipoate or some closely related substance. Thus, the oxidation of succinate requires its prior "activation" to allow it to reach the oxidation systems. We believe that the "activation" step is catalyzed by an enzyme located in the plasma membrane.

Hess: I would like to ask a short question to one of the last three speakers about the arsenate treatment; according to Dr. Ernster the pyridine nucleotides were largely reduced after the arsenate treatment. How do you explain it? And what is the reductant?

PACKER: I would like to comment on some recent experiments which Dr. E. E. Jacobs and I have been doing, as they may have some bearing on this problem. We have been looking at certain shunts of electron transport involving the oxidase end of the chain. For example, we use ascorbate as reducing agent and catalysts such as tetramethylphenylenediamine; we have been able to show that these shunts can be tightly coupled to phosphorylation and show respiratory control with ADP and thus can calculate P to O ratios by measuring O_2 utilization (polarographically). If the mitochondria are carefully washed the endogenous substrate can be removed so that P:O ratios come out to be about 1.0. It happens that this shunt is capable of reducing pyridine nucleotides, and in the steady state the pyridine nucleotides can be oxidized and reduced by initiating a brief cycle of phosphorylation by adding a small amount of ADP. The oxidation through the shunt is not inhibited by antimycin but the reduction of pyridine nucleotide is completely blocked in the presence of this substance. I would commend this shunt as of possible interest in connection with the phenomenon of reversal of electron transport just discussed.

ESTABROOK: I should just like to state that in particles which we know are devoid of endogenous substrate, DNP preincubation does not inhibit succinate oxidation. This is a situation with particles which will give good phosphorylation in the absence of DNP and also a situation where pyridine nucleotide is reduced by succinate. This is one more additional piece of evidence for the complexity of the endogenous substrate.

AZZONE: I don't see why this should be.

ESTABROOK: You didn't produce any oxaloacetate.

CHAPPELL: The same is true in kidney mitochondria which has very little endogenous substrate.

AZZONE: We think that the ATP requirement for succinate oxidation is present only above a certain level of structural integrity. If we take away, by destroying or fragmenting the mitochondria, the structural barrier which makes necessary the energy-requiring reaction then we find neither an inhibition nor an ATP stimulation of succinate oxidation.

CHAPPELL: This is not true in kidney mitochondria which responds to normal respiratory control and P:O ratios, and they are just free of endogenous substrate. The addition of oxaloacetate or malate will induce all the phenomena which you

have just talked about. We can see the same with malate in the digitonin-extracted particles.

WILLIAMS: Can I just make one more point because I think this is going to be misunderstood by some people, the question is not whether there is an energy-linked reduction of pyridine nucleotide but the relation of this reduction to respiration, and to this Dr. Packer's data are totally irrelevant although of great intrinsic interest.

Pyridine Nucleotides in Mitochondria

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One of the experimental difficulties in studying the mechanism of respiratory-chain phosphorylation is that the concentration of the high-energy phosphate compounds, which must surely be intermediates in this reaction, will be of the same order as that of the mitochondrial enzyme-coenzyme system which catalyzes the reaction. This situation may be contrasted with that appertaining to glycolytic phosphorylation, where the high-energy phosphate intermediate (diphosphoglyceric acid) can accumulate in amounts of the same order as the substrate concentration.

This difficulty can be decreased by about one order of magnitude by studying those components of the respiratory chain which are present in the greatest concentration in the mitochondria, viz. the pyridine nucleotides and ubiquinone (coenzyme Q). This lecture is concerned with the pyridine nucleotides.

It is only during the last 10 years or so that the pyridine nucleotides have been considered to be constituents of the mitochondria. In the nineteen-thirties, diphosphopyridine nucleotide at least was thought of as a dissociable coenzyme catalyzing anaerobic oxido-reductions in the "soluble" fraction of the cytoplasm. The first indication that it was much more firmly bound came from the isolation by Cori in 1948 of crystalline phosphoglyceraldehyde dehydrogenase containing firmly bound DPN^+ [1]. Even more important for the topic of this lecture was the observation of Huennekens and Green [2] that rabbit-liver and rabbit-kidney "cyclophorase" preparations, consisting largely of mitochondria, contained considerable amounts of firmly bound pyridine nucleotide, sufficient for maximal respiration in the absence of added pyridine nucleotide. The amounts of the pyridine nucleotide were little changed after prolonged incubation in the presence of substrates. The importance of the mitochondrial pyridine nucleotides was further stressed by Lehninger's [3] observation that extra-mitochondrial DPNH was only very slowly oxidized

by intact liver mitochondria, and by Christie and Judah's [4] similar finding with respect to reduction of DPN.*

The intramitochondrial pyridine nucleotides are also inaccessible to DPN⁺-destroying enzymes† which are present in the microsome fraction [5]. The fact that the pyridine nucleotides are retained within the mitochondria, even when the mitochondrial suspension is diluted, provides the opportunity of studying the oxidation and reduction of the mitochondrial pyridine nucleotides during the operation of oxidative phosphorylation, an opportunity availed of with great success by Chance and Williams [7], and by Klingenberg and his co-workers [8, 9].

For some time, we, like others, have been interested in the possibility that a compound of DPN with some substance, variously known as C, I or X, might be an intermediate in chemical reactions which link intracellular respiration with the synthesis of ATP. About 2 years ago, Dr. Purvis announced from our laboratory that he had evidence that rat-liver mitochondria contained, besides DPN⁺ and DPNH, a third form of diphosphopyridine nucleotide, termed "extra DPN", which accumulated in and disappeared from the mitochondria in a manner that might be expected for an intermediate of oxidative phosphorylation [10, 11]. This conclusion has been criticized by others, whose experiments gave no evidence of a diphosphopyridine nucleotide compound other than DPN⁺ and DPNH.

During the last 2 years, Dr. Purvis has been continuing this investigation in Dr. Kaplan's laboratory in Brandeis, while we have been following up other aspects in Amsterdam. Although our studies are not yet completed, I thought it only fair to other workers in the field that we bring out an interim report of our experiments.

Pyridine nucleotide content of isolated mitochondria

Huennekens and Green's [2] measurements of the pyridine nucleotide contents of cyclophorase preparations were followed by determinations by Holton [12] of the DPN⁺ content of isolated rat-heart sarcosomes, and by Glock and McLean [13] and Jacobson and Kaplan [14] of the DPN⁺, DPNH, TPN⁺ and TPNH content of isolated rat-liver mitochondria. The

* Birt and Bartley [6] have recently confirmed by direct analysis that mitochondria can exclude added DPN⁺ and DPNH from participation in intramitochondrial processes of oxidation and reduction. TPN⁺ and TPNH, on the other hand, can enter the mitochondria readily.

† Abbreviations: DPN⁺, DPNH, oxidized and reduced diphosphopyridine nucleotide; DPN, all forms of diphosphopyridine nucleotide; TPN⁺, TPNH, TPN, corresponding symbols for compounds of triphosphopyridine nucleotide; ADP, ATP, adenosine di- and triphosphate; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetate.

TABLE I
PYRIDINE NUCLEOTIDE CONTENT OF RAT-LIVER MITOCHONDRIA
μmoles/g. protein

Author	Method*	Means (no. of preparations in brackets)							
		DPN +	DPNH	DPN ++ DPNH	Total DPN*	TPN +	TPNH	TPN ++ TPNH	Total TPN**
Glock and McLellan [13]	C	1.2	0.7	1.9	—	0.2	1.6	1.8	—
Jacobson and Kaplan [14]	F	1.4	0.6	2.0	—	0.4	3.9	4.4	—
Klingenberg and Slenczka [8]	S	0.7 (60)	1.2 (60)	1.9	—	0.2 (60)	3.2 (60)	3.4	—
Birt and Bartley [6]	F	3.0 (17)	0.7 (17)	3.7	—	1.9 (17)	1.6 (17)	3.5	—
Purvis§§	F	1.5 (14)	1.3 (14)	2.8	4.1 (14)	0.3 (13)	3.3 (13)	3.6	5.0 (13)
<i>Amsterdam</i>									
Myers†	S	2.0 (3)	—	—	4.5 (3)	—	—	—	—
Purvis§	F	1.4 (49)	1.7 (49)	3.1	4.2 (49)	0.2 (18)	2.3 (18)	2.5	5.0 (18)
Bouman	F	1.4 (30)	2.7 (37)	4.1	4.2 (21)	0.4 (2)	4.2 (6)	4.6	—
Winter	F	1.4 (20)	2.3 (26)	3.7	3.7 (13)	0.3 (4)	4.9 (13)	5.2	—
Baillie	S	1.7 (16)	2.4 (16)	4.1	3.9 (10)	0.2 (3)	5.0 (4)	5.2	—
	F	1.7 (4)	2.2 (4)	3.9	4.1 (4)	—	—	—	—

* F = enzymic fluorimetric; S = enzymic spectrophotometric; C = enzymic catalytic method.

† Published in Holton *et al.* [15].

‡ Mitochondria prepared in Brandeis.

§ Published in Purvis [11].

|| Unpublished.

** Determined by incubation with P_i, ADP + P_i, or dinitrophenol [11].

TABLE II
 PYRIDINE NUCLEOTIDE CONTENT OF HEART SARCOMERES
 μ moles g. protein

Author	Animal	Method*	Means (no. of preparations in brackets)							
			DPN ⁺	DPNH	DPN ⁺⁺ DPNH	TPN ⁺	TPNH	TPN ⁺⁺ TPNH	Total TPN ⁺⁺	
Holton [12]	Rat	S	6.0 (2)	—	—	—	—	—	—	—
Holton <i>et al.</i> [15]	Rat	S	6.4 (2)	—	—	—	—	—	—	—
Jacobson and Kaplan [14]	Rat	F	1.4	0.1	1.5	—	—	—	—	—
Purvis†	Rat	F	—	—	6.5 (2)	—	—	—	—	—
Lester and Hatefi [39]	Ox	S	—	—	—	5.6 (3)	—	—	—	0.9 (3)
Klingenberg <i>et al.</i> [9]	Rat	S	—	—	4.2 (10)	—	—	—	—	1.0 (10)
Bailie†	Rabbit	F	4.6 (11)	1.4 (11)	6.0	7.6 (11)	0.6 (1)	0.7 (1)	1.3	1.6

* F = enzymic fluorimetric; S = enzymic spectrophotometric; C = enzymic catalytic method.

† Unpublished.

‡ Determined by incubation with P_i, ADP + P_i, or dinitrophenol [11].

latter two groups found unexpectedly high concentrations of TPNH. The mean values obtained by these authors, and in later studies, are listed in Table I (rat liver) and Table II (heart).

In Table I are shown the values obtained in the Amsterdam laboratory over the last 4 years by five different workers. DPN⁺, TPN⁺ and DPNH, TPNH were determined in acid and alkali extracts, respectively, of the freshly prepared mitochondria. Total DPN and TPN refer to the amounts of oxidized nucleotides found when the mitochondria were treated in such a way as to convert all the pyridine nucleotides into the oxidized form (see below). The total DPN and TPN contents of our preparations have remained almost constant during this period. Purvis has also obtained the same values for mitochondria prepared by our procedure in Brandeis.

The Amsterdam values for the total DPN content are quite similar to those reported by Birt and Bartley [6] for DPN⁺⁺DPNH, but are considerably higher than the others in Table I.* Our total TPN values are rather higher than the (TPN⁻+TPNH) measured by other workers. It is not known to what extent these differences represent differences in the nutritional status of the rats used, or in the methods used to isolate the mitochondria. All workers report considerable variation from preparation to preparation.

With respect to the DPNH/DPN⁺ and TPNH/TPN⁺ ratios our preparations closely resemble those of Klingenberg and Slenczka [8]. Birt and Bartley's preparations contain much more of the oxidized pyridine nucleotide. This difference is probably connected with the method of preparation of the mitochondria.†

Miss Baillie has found essentially the same values by our enzymic fluorimetric procedure, which is similar to that used by Jacobson and Kaplan [14] and Purvis [11], and by a spectrophotometric method, which differs somewhat from others described in respect to the determination of the reduced pyridine nucleotides. The alkali extract is neutralized, treated with α -ketoglutarate, NH_4^+ and glutamate dehydrogenase to oxidize the DPNH and TPNH, and deproteinized with HClO_4 . DPN⁺ and TPN⁺ are determined spectrophotometrically on the completely deproteinized solution by successive additions of ethanol+alcohol dehydrogenase (at pH 10) and isocitrate and isocitrate dehydrogenase (at pH 7.4). The spectrophotometric procedure is rather more reproducible but less sensitive than the fluorimetric.

* Klingenberg *et al.* [9] have suggested that the method used by Holton *et al.* [15] to determine the DPN⁺ content of rat-liver mitochondria might also estimate TPN⁺, owing to traces of TPN⁺-specific alcohol dehydrogenase in our preparations of this enzyme. This was not the case. TPN⁺ is not estimated either in the procedure used by Holton *et al.* [15] or in our recent work (see Purvis [11], Table I).

† A more recent paper (*Biochem. J.* **76**, 328 (1960)) reports more of the reduced nucleotides.

Oxidation and reduction of mitochondrial pyridine nucleotide

The first study of the oxidation and reduction of pyridine nucleotides in mitochondria was carried out by Chance and Williams [7], who made the important discovery that, in the presence of substrate, the pyridine nucleotides were largely reduced when the respiration of the mitochondria was "inhibited" owing to lack of ADP, and were oxidized by the additions of ADP. These measurements were made by a sensitive spectrophotometric technique, which enabled the determination of the absorbancy

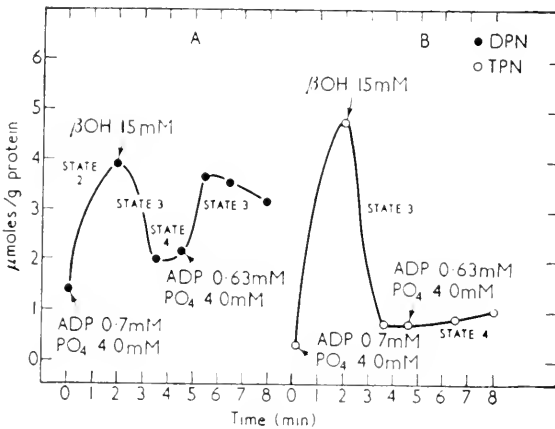


FIG. 1. DPN⁺ and TPN⁺ contents of rat-liver mitochondria in different "States" [7]. Rat-liver mitochondria (final concn. 2.8 mg. protein/ml.) were suspended in 0.21 M sucrose, 33 mM nicotinamide, 2.5 mM MgCl₂ final volume 2.7 ml. The following additions were made: at zero time, 0.7 mM ADP and 4.0 mM P_i; at 2 min., 15 mM β-hydroxybutyrate; at 4.5 min., 0.63 mM ADP and 4.0 mM P_i. DPN⁺ and TPN⁺ were determined on aliquots of the same suspension. Abbreviations: PO₄, P_i; βOH, β-hydroxybutyrate. Temperature, 0°. Unpublished experiment of Dr. J. L. Purvis.

changes at 340 mμ in a mitochondrial suspension. Chance and Williams [16] interpreted these absorbancy changes as reflecting changes in the oxido-reduction state of DPN. This became uncertain when Glock and McLean [13] and Jacobson and Kaplan [14], by specific enzymic tests on deproteinized extracts, showed that rat-liver mitochondria contained much more TPNH than DPNH. However, a recent study by Klingenberg and Slenczka [8], who used both Chance's method of direct spectrophotometric observation of the mitochondrial suspension and the less sensitive but more specific enzymic assays on deproteinized extracts, have confirmed the most important findings of Chance and Williams. In particular, they showed that the rapid absorbancy changes which follow exhaustion or

TABLE III

OXIDATION OF DPNH BY INCUBATION OF RAT-LIVER MITOCHONDRIA UNDER VARIOUS CONDITIONS

Unpublished experiments of J. Bouman (Expts. 1 and 2), and of M. Baile (Expt. 3). Incubation at room temperature (20-25°).

Expt.	[Sucrose] (M)	[Dinitrophenol] (mM)	[P _i] (mM)	[ADP] (mM)	-ΔDPN ⁺ (μmoles g. protein) after		
					2 min.	5 min.	10 min.
1*	0.25	—	—	—	—	0.44	—
	0.25	0.1	—	—	—	2.35	—
	0.05	—	—	—	—	2.18	—
	0.05	—	50	—	—	2.80	2.77
2	0.16	—	—	—	0.04	—	—
	0.16	0.01	—	—	1.68	2.51	—
3 (P ₀₄₈)	0.15	—	50	—	—	—	1.70
	0.25	—	1.6	1.6	—	—	1.44

* Mean of 3 experiments.

addition of ADP are indeed due to changes in the oxidation-reduction state of DPN, changes in TPN occurring more slowly. The finding of TPNH in the mitochondria does, it is true, necessitate rather important quantitative changes in the interpretation given by Chance and Williams, e.g. whereas the latter concluded that transition from State 3 (active respiration) to State 4 (respiration "inhibited" or controlled owing to lack of ADP) was associated with a change in DPN from 50% to 99% reduced, Klingenberg and Slenczka found that the corresponding values were 3% and 43% reduced. Thus, even in the controlled state there was considerable DPN in the oxidized form.

Purvis (unpublished observations) has obtained similar results, which are given in Fig. 1. Addition of ADP and P_i to a mitochondrial suspension causes oxidation of the reduced nucleotides due to exhaustion of endogenous substrate. After addition of substrate (β -hydroxybutyrate) the ADP is soon all phosphorylated, so that the mitochondria reach State 4, in which about 51% of the total DPN (measured in State 2) and 6% of the total TPN are in the oxidized form. Addition of more ADP brings the mitochondria into State 3, in which 94% of the DPN is oxidized. On the other hand, there was little formation of TPN⁺. These results confirm three of Klingenberg and Slenczka's findings, viz. (a) DPN is not completely reduced in State 4; (b) in both States 3 and 4 the predominant reduced pyridine nucleotide is TPNH; (c) DPNH responds to addition of ADP much more rapidly than TPNH. As Klingenberg *et al.* [9] have pointed out, the presence of large amounts of DPN⁺ in the controlled state removes one of the main arguments of Chance and Williams [7] that DPNH is present in an inhibited form (DPNH \sim I).

The high concentrations of reduced pyridine nucleotides in freshly prepared liver mitochondria (cf. Table I) are presumably caused by the presence of endogenous substrate and an "inhibited" respiration, owing to the absence of ADP. The DPNH is only quite slowly oxidized when the preparation is diluted in isotonic medium, or near isotonic (see Table III—cf. Kaufman and Kaplan [5]), but it is rapidly oxidized in hypotonic medium without further addition, or in isotonic medium by addition of dinitrophenol, 0.05 M P_i or lower concentrations of P_i in the presence of ADP (Table III and Fig. 1—cf. refs. [7, 17, 8, 11, 5]).

Purvis' "extra DPN"

One of the most important questions is whether the pyridine nucleotide present in the mitochondria is free or bound. Huennekens and Green [2] concluded that it was not free and that "the principal oxidases of the cyclophorase complex occur as conjugated pyridinoprotein enzymes". However, the concept that mitochondria were bound by a semi-permeable

membrane was not then accepted by these workers. Because the intramitochondrial DPNH could not be oxidized in the inhibited State 4, Chance and Williams [7] and Chance and H. Baltscheffsky [18] concluded that DPNH was in an inhibited form $DPNH \sim I$. Chance and Hollunger [19] have recently stated, "It is further concluded that the reduced form is bound to some ligand, for example, a protein, because the fluorescence maximum is at $443 m\mu$. Thus this material is denoted $DPNH \sim I$"

It is doubtful whether the sort of chemical bonds which Huennekens and Green [2] and Chance and Hollunger [19] have in mind could survive the treatment with acid or alkali used to prepare deproteinized extracts for determinations of the pyridine nucleotides. It is probable, therefore, that these bound forms would yield free DPN^+ and DPNH in the extracts. We were more concerned with the possibility that a stable compound of DPN of low molecular weight, which could survive either the acid or alkali extraction, might be present in mitochondria.

Purvis [10, 11] found that the total amount of DPN and TPN, determined by incubation of rat-liver mitochondria with P_i , $ADP + P_i$, or dinitrophenol, appreciably exceeded the amounts of $(DPN^+ + DPNH)$ and of $(TPN^+ + TPNH)$, respectively, determined in the fresh mitochondria (see Table I). The amount of "extra DPN" found in this way averaged $1.10 \mu\text{moles/g. protein}$ for the Amsterdam preparations and $1.37 \mu\text{moles/g. protein}$ for the Brandeis preparations. The corresponding values for "extra TPN" were 2.50 and 1.42, respectively. Only two out of sixty-three preparations examined did not show any of this material.

Klingenberg and Slenczka [8], who did not find any evidence for "extra DPN", in somewhat different experiments, concluded that Purvis' results were due to a failure of his fluorimetric procedure.

Table I shows that we also, on the average, find little if any excess of

* The difference spectrum (anaerobic minus aerobic steady state) shows a pyridine nucleotide peak at $320 m\mu$ rather than at $340 m\mu$ [16 (see Fig. 1), 15, 17]. This does not, however, prove that the DPNH found in the mitochondria is "bound" in such a way as to cause a displacement of the absorption peak, as unfortunately appears to be implied by Holton *et al.* [15]. Bücher and Klingenberg [20] have with justification criticized this conclusion, which in fact we had not intended should be made from our results, since the displacement of the $340\text{-}m\mu$ peak might be caused by the contribution of the δ -bands of the cytochromes in this region of the spectrum, as already discussed by Holton [12]. Chance and M. Baltscheffsky [17], Chance [21], and Chance and Hollunger [19] have pointed out that the DPNH which appears when ADP is exhausted by a respiring mitochondrial preparation has an absorption peak at $340 m\mu$. The changes in the degree of reduction of the cytochromes is much less than that of the DPN under these conditions, so that the contribution of the cytochrome δ -bands to the spectrum in the near ultraviolet would be much less. It should be pointed out, however, that Chance and M. Baltscheffsky [17] are of the opinion that the displacement of the DPNH peak in the anaerobic spectrum cannot be explained by the δ -bands of the cytochromes.

"total DPN" over and above (DPN⁺ + DPNH). Since, however, we have used Purvis' procedure with only minor modifications, we did not think it so likely that his results were due to an analytical error. A few prepara-

TABLE IV
PERCENTAGE OF RAT-LIVER MITOCHONDRIA PREPARATIONS CONTAINING
"EXTRA DPN"

	Purvis [11]	Present work
No. of preparations	63	48
<i>Percentage with "Extra DPN"</i>		
less than — 0.4 μ mole/g. protein	0	10
between — 0.4 and 0 μ mole/g. protein	3	46
0 and 0.4 μ mole/g. protein	6	23
0.4 and 0.8 μ mole/g. protein	14	12
more than 0.8 μ mole/g. protein	76	8
	—	—
	99	99

TABLE V

EFFECT OF ADDED SUBSTRATE (90 SEC. AT 0) ON FORMS OF DIPHOSPHOPYRIDINE NUCLEOTIDE IN RAT-LIVER MITOCHONDRIA

Mitochondria suspended in 0.18 M sucrose. Unpublished results of Dr. J. L. Purvis, some of which were reported by Slater and Hülsmann [29]. Values in μ moles/g. protein.

Expt.	Substrate	DPN ⁺	DPNH	"Extra DPN"*
1	None	1.78	1.56	1.10
	α -Ketoglutarate (20 mM)	0.70	1.40	2.34
2	Glutamate (40 mM)	1.28	1.56	1.60
	None	1.60	1.94	1.18
3	Succinate (40 mM)	0.82	1.77	2.13
	None	1.73	2.51	0.70
4	Succinate (40 mM)	0.74	2.16	2.00
	None	1.55	1.50	1.42
5	Fumarate (20 mM)	1.99	1.30	1.21
	None	1.74	1.23	0.97
	Malate (20 mM)	1.64	1.39	0.91

* Total DPN *minus* (DPN⁺ + DPNH). Total DPN determined by incubation in absence of substrate with dinitrophenol at 30°.

tions gave results resembling those of Purvis. Table IV shows that whereas Purvis found that 90% of his preparations contained more than 0.4 μ mole/g. protein "extra DPN", only 20% of our preparations contained

this amount of "extra DPN". Although we are under the impression that some of our preparations of liver mitochondria have the properties ascribed to them by Purvis, they turn up so rarely that we could not be absolutely certain that they were not due to analytical errors.

Further evidence for the presence in liver mitochondria of a form of diphosphopyridine nucleotide other than DPN and DPNH was obtained by Purvis by studying the effect of adding substrates. Table V shows that the addition of α -ketoglutarate, glutamate or succinate for 90 sec. at 0° (without added substrate there is no change in the amount of DPN⁺ and DPNH during this period) caused a decline in the amount of DPN⁺, without any increase in the amount of DPNH, i.e. some pyridine nucleotide disappeared. Malate and fumarate, on the other hand, caused little change in the DPN⁺ or DPNH level.

TABLE VI

EFFECT OF ADDED SUCCINATE (90 SEC. AT 0°) ON FORMS OF DIPHOSPHOPYRIDINE NUCLEOTIDE IN RAT-LIVER MITOCHONDRIA

Unpublished experiments of J. Bouman, B. Winter and M. Bailie. Mitochondria suspended in 0.18-0.25 M sucrose. Value in μ moles/g. protein.

Method	No. of expts.	Δ DPN ⁺	Δ DPNH
<i>A. Mean results</i>			
Fluorimetric	11	-0.61	+0.66
Spectrophotometric	4	-0.94	+0.60
<i>B. Single experiment (spectrophotometric method)</i>			
	DPN ⁻	DPNH	"Extra DPN"*
Fresh mitochondria	2.34	1.54	0.57
+ Succinate (40 mM)	0.81	2.28	1.36

* Total DPN (determined by incubation in absence of substrate with 0.05 M P_i for 10 min.) minus (DPN⁻ + DPNH).

The results with succinate appeared to be in conflict with those of Chance and Hollunger [22], who reported extensive reduction of DPN⁺ by this substrate.

When we repeated these experiments with our preparations of liver mitochondria, which contained little "extra DPN", the DPN⁺ which disappeared nearly always appeared as DPNH (see Table VI*A*). These results support Chance and Hollunger [22], whose findings have in the meantime been confirmed by Klingenberg *et al.* [9]. As a whole, they give no support to the existence of another form of DPN. When, however, succinate was added to one of our rare preparations which appeared to contain "extra DPN", a result was obtained intermediate between that

reported by Purvis and the bulk of our results, in that the DPNH content increased, but not to the same extent as the decrease of DPN^+ (Table VIB). This result suggests the possibility that, whenever we have a preparation of fresh rat-liver mitochondria containing some "extra DPN", we can increase the amount by adding succinate. However, it must be emphasized that Table VIB describes a single result, which has not yet been reproduced.

At this stage, we must conclude that the tightly-coupled rat-liver mitochondria which we normally prepare in Amsterdam rarely contain "extra DPN", and that this substance cannot usually be induced by a short-term incubation with succinate. Further progress clearly required a reproducible method of inducing the "extra DPN".

TABLE VII

EFFECT OF INCUBATION WITH GLUTAMATE OR SUCCINATE IN P_i -DEFICIENT MEDIUM, AND OF THE SUBSEQUENT ADDITION OF DINITROPHENOL ON DPN^+ AND DPNH CONTENTS OF RAT-LIVER MITOCHONDRIA

Unpublished experiments of B. Winter and M. Bailie. Mean values ($\mu\text{moles/g. protein.}$)

Substrate:	Glutamate	Glutamate	Succinate
Analytical Method:	Fluorimetric	Spectro- photometric	Fluorimetric
No. of expts.:	8	4	3
<i>Changes on incubation with substrate</i>			
ΔDPN^+	-0.09	-0.68	-0.47
ΔDPNH	-0.63	+0.23	+0.06
$\Delta(\text{DPN}^+ + \text{DPNH})$	-0.72	-0.45	-0.41
<i>Changes on subsequent addition of dinitrophenol</i>			
ΔDPN^+	1.88	1.91*	1.57
ΔDPNH	-0.79	-1.85	-1.20
$\Delta(\text{DPN}^+ + \text{DPNH})$	1.09	0.06*	0.37

* Means 2.08 and 0.23, respectively, if one doubtful value is omitted.

If the "extra DPN" is really an intermediate in oxidative phosphorylation, as we hope, we should expect that it would accumulate in the absence of inorganic phosphate or ADP. For this reason, we tried incubation with glutamate in the presence of ADP, hexokinase, glucose, and nicotinamide. Under these conditions, any traces of inorganic phosphate are rapidly esterified and accumulate as hexose monophosphate. The rate of respiration is only about one-seventh that obtained on the addition of inorganic phosphate [23, 24].

Figure 2 (A) and the second column of Table VII show the results of a

series of eight such experiments with rat-liver mitochondria. On the average, there was little change in the DPN^+ content while the DPNH declined. As a consequence, the $(\text{DPN}^+ + \text{DPNH})$ content declined in every experiment, by an average of $0.73 \mu\text{mole g. protein}$. The amount of total diphosphopyridine nucleotide which disappeared varied considerably from experiment to experiment—between 0.2 and $1.5 \mu\text{moles/g. protein}$. It exceeded $0.4 \mu\text{mole g. protein}$ in six of the eight experiments.

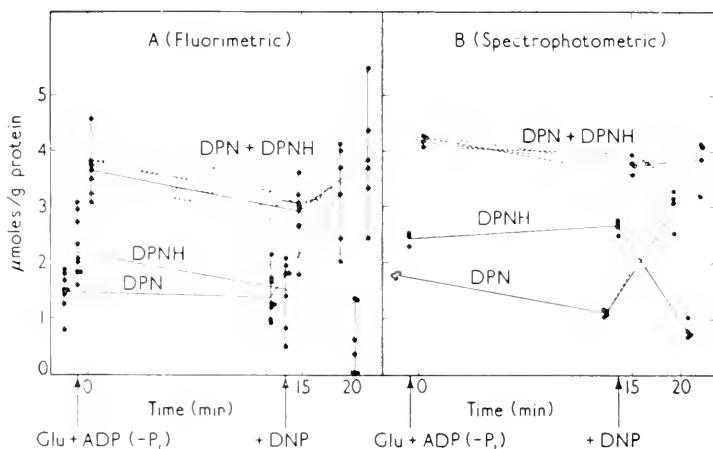


FIG. 2. DPN^+ and DPNH contents of rat-liver mitochondria after incubation with glutamate in the absence of P_i and after adding dinitrophenol.

A. A set of eight experiments, in which the fluorimetric enzymic assay was used. The first series of vertical lines show the range of values for the DPN^+ , the DPNH and the $\text{DPN}^+ + \text{DPNH}$ contents of the fresh mitochondria. The points show the individual values. At zero time the mitochondrial suspension was added to a mixture containing 13.3 mM KCl , 1.8 mM EDTA , 13.5 mM glucose , 4.5 mM MgCl_2 , 9 mM L-glutamate , 0.8 mM ADP , $38.7 \text{ mM nicotinamide}$, 0.15 M sucrose (derived from the mitochondrial suspension) and hexokinase. The final concentration of mitochondrial protein was about 12 mg. ml . After 15 min. in a manometer flask the suspensions were analyzed for DPN^+ and DPNH . The values are shown on the second set of vertical lines. Duplicate flasks were treated in the same way, until at 15 min. $0.1-1 \text{ mM}$ dinitrophenol was added from the side-arm. After a further 5 min. the DPN^+ and DPNH contents of these suspensions were determined. The values are shown in the third set of vertical lines. The full lines connecting these vertical lines show the changes in the mean values of DPN^+ , DPNH and $\text{DPN}^+ + \text{DPNH}$. The discontinuous lines show the changes in $(\text{DPN}^+ + \text{DPNH})$ in the individual experiments.

B. A set of four experiments, in which the spectrophotometric enzymic method was used. Conditions and reaction mixture as in A, except that a concentration of about $18 \text{ mg. mitochondrial protein ml}$. was used. The full lines connect the mean values of DPN^+ and DPNH . The discontinuous lines show the change of $\text{DPN}^+ + \text{DPNH}$ in each experiment. One of the DPN^+ values after dinitrophenol is probably a mistake. The dotted line is drawn to the mean obtained by ignoring this value.

It should be emphasized that all of these experiments were carried out in the presence of nicotinamide, which prevents the destruction of DPN^+ . In six of the eight experiments, we added dinitrophenol which, in agreement with Chance's observations with other uncoupling agents, brought about oxidation of much of the DPNH . There was an increase in the $(\text{DPN}^+ + \text{DPNH})$ content of between 0.3 and 2.4 (average 1.1) $\mu\text{moles/g.}$ protein.

Thus, during the incubation with substrate a part of the total DPN disappears into a compound which does not react in our enzymic method. It reappears as DPN^+ on addition of dinitrophenol. This compound is not TPN^+ . In three experiments, in which an average of 1.1 μmole $(\text{DPN}^+ + \text{DPNH})/\text{g.}$ protein disappeared during incubation, 0.25 μmole $(\text{TPN}^+ + \text{TPNH})/\text{g.}$ protein also disappeared. In four experiments, 0.3 μmole $(\text{TPN}^+ + \text{TPNH})/\text{g.}$ protein appeared after addition of the dinitrophenol.

Recently, this experiment has been repeated with four preparations using a spectrophotometric method. There is much less variation, which may be partly due to the greater accuracy of the spectrophotometric assay, but is also probably due to the fact that the experiments were carried out in close succession with mitochondrial preparations which were probably very similar to one another. Qualitatively, the same picture is shown (see Fig. 2 (B)), but the amount of total pyridine nucleotide disappearing was rather less—between 0.2 and 0.7 $\mu\text{mole/g.}$ protein.

Three experiments (fluorimetric) were also carried out with succinate in place of glutamate. There was some loss of DPN^+ , while the DPNH content did not increase (see column 4, Table VII).

From these experiments, we can conclude that incubation with substrate in the absence of inorganic phosphate causes some of the diphosphopyridine nucleotide to disappear, and that what disappears can be largely recovered again by the addition of dinitrophenol. The amount of DPN disappearing in this way was sometimes quite large, but was often only small in experiments which appeared to be carried out in the same way with identical material. We do not understand the reasons for these differences and, at present, our preparations of rat-liver mitochondria are discouraging material for the study of the Purvis compounds. For this reason, we have recently turned to other mitochondria.

However, before leaving the experiments summarized in Fig. 2 and Table VII, it is worth while drawing attention to the state of oxidation of the pyridine nucleotides in the controlled and active states. In these experiments, respiration was controlled or inhibited by lack of inorganic phosphate. In this controlled state, a substantial proportion of the diphosphopyridine nucleotide is in the oxidized form, nearly half in the first series of experiments (Fig. 2 (A)). This is similar to Klingenberg and Slenczka's finding when respiration was inhibited by lack of ADP . In fact,

we found very similar results with glutamate as substrate whether lack of P_i or ADP was responsible for the inhibition of respiration (see Table VIII). In the controlled state, the degree of oxidation of the pyridine nucleotides is probably largely controlled by equilibria catalyzed by the DPN-specific dehydrogenases, such as



Adding dinitrophenol to a mitochondrial suspension in the presence of glutamate and absence of P_i causes about a 3-fold increase in the rate of O_2 uptake [23]. This is presumably due to activation of DPNH oxidation, with a consequent increase in the rate of oxidation of glutamate to α -ketoglutarate. Further oxidation of the α -ketoglutarate cannot occur at an

TABLE VIII

DIPHOSPHOPYRIDINE NUCLEOTIDE COMPOUNDS OF RAT-LIVER MITOCHONDRIA IN CONTROLLED STATE

Glutamate substrate. The P_i -deficient medium was the same as that in Fig. 2. The ADP-deficient medium contained 15 mM KCl, 5 mM $MgCl_2$, 30 mM nicotinamide, 40 mM tris(hydroxymethyl)aminomethane—HCl buffer, pH 7.4, 40 mM potassium phosphate, pH 7.4, 2 mM EDTA, 0.1 mM ADP, 0.12 M sucrose, 30 mM glutamate. Single experiment (fluorimetric). Values in μ moles/g. protein. Unpublished experiment of M. Bailie.

State of mitochondria	DPN ⁺	DPNH	"Extra DPN"*
Fresh	1.51	2.30	0.54
In P_i -deficient medium	1.72	1.89	0.74
In ADP-deficient medium	1.88	1.83	0.64

* Determined as in Table VI.

appreciable rate, because P_i is necessary for the substrate-linked phosphorylation step of α -ketoglutarate oxidation even in the presence of dinitrophenol [25, 26]. The marked decrease in the DPNH concentration shown in Fig. 2 is to be expected.

In other experiments, not shown in Fig. 2, respiration was fully activated (sevenfold) by adding P_i instead of dinitrophenol. This not only activates DPNH oxidation, but also DPN⁺ reduction by α -ketoglutarate and by malate (the oxidation of malate to oxaloacetate is involved in the oxidation of glutamate by mitochondrial preparations [27]). In fact, the sevenfold stimulation of the respiratory rate was accompanied by very little change in the degree of oxidation of the diphosphopyridine nucleotide (means of four experiments: Δ DPN⁺, +0.35 μ mole/g. protein; Δ DPNH, -0.21 μ mole/g. protein). Thus, under the conditions of our experiments, the diphosphopyridine nucleotide of rat-liver mitochondria oxidizing glutamate in the presence of P_i and ADP is about 50% reduced. This is

much greater than the degree of reduction reported by Klingenberg *et al.* [9], viz. 1-10% reduced. No doubt, a difference in experimental conditions is responsible for this discrepancy.

Sarcosomes

Muscle mitochondria (sarcosomes) have been found to be a much more reproducible source of "extra DPN". Out of eleven preparations of

TABLE IX
"EXTRA DPN" IN SARCSOMES

Unpublished experiments of M. Bailie. Values in μ moles/g. protein.

Expt.	DPN ⁺	DPNH	"Extra DPN"*	"Extra DPN"* after incubation with substrate†	
				in ADP-deficient medium‡	in P _i -deficient medium‡
<i>Rabbit heart</i> (fluorimetric method)					
E042	6.2	0.4	1.8	1.5	—
E044	6.4	0.6	-0.2	1.3	—
E047	4.7	2.1	1.9	2.1	—
E053	5.5	1.1	1.3	2.6	—
E055	4.2	1.8	2.3	3.1	—
E057	4.4	1.7	1.5	—	—
E065	3.5	2.1	0.8	—	—
E066	4.5	1.5	1.3	—	—
E067	5.3	1.0	3.8	—	—
E068	3.9	1.0	1.7	—	—
E071	2.8	1.6	1.4	2.3	3.2
<i>Means</i>	<i>4.6</i>	<i>1.4</i>	<i>1.6</i>	<i>2.2</i>	<i>(3.2)</i>
<i>Pigeon breast</i> (spectrophotometric method)					
E063	5.2	1.0	0.6	—	—
E070	4.5	1.5	3.1	2.5	4.0
E071	3.7	3.0	1.2	—	—
E072	5.5	0.7	0.4	—	—
<i>Means</i>	<i>4.7</i>	<i>1.5</i>	<i>1.7</i>	—	—

* Determined as in Table VI.

† Succinate (40 mM), or succinate (40 mM) + glutamate (30 mM) as substrate.

‡ Composition as in Table VIII.

rabbit-heart sarcosomes, and four of pigeon-breast, only one preparation was found not to contain any "extra DPN" (see Table IX). The amount was increased by incubation with succinate in ADP-deficient medium

(State 4) and especially in a P_i -deficient medium. The one preparation (Eo44) without "extra DPN" contained it after incubation with succinate. It is noteworthy that, in disagreement with our finding with rat-liver mitochondria (Table VIII), more "extra DPN" was found on incubation of heart sarcosomes in P_i -deficient medium than in ADP-

TABLE X

REDUCTION OF DPN⁺ BROUGHT ABOUT BY ADDING SUCCINATE TO ADP-DEFICIENT RABBIT-HEART SARCSOMES IN PRESENCE OF GLUTAMATE

Unpublished experiments of M. Bailie. Values in μ moles/g. protein.

Expt.		Eo42	Eo44	Eo47
DPN ⁺ ,	before succinate	6.57	4.07	7.35
	after succinate	3.31	1.82	2.74
JDPN ⁺		-3.26	-2.25	-4.61
DPNH,	before succinate	0.30	1.22	1.19
	after succinate	3.62	3.21	3.82
JDPNH		3.32	1.99	2.63

deficient medium. This is probably related to the fact that the degree of respiratory control is much greater with sarcosomes oxidizing succinate in a P_i -deficient medium than in an ADP-deficient medium.

The "extra DPN" is not derived from TPN. In Expt. o66, the freshly prepared sarcosomes contained TPN⁺, 0.58; TPNH, 0.73; "extra TPN", 0.26 μ mole/g. protein.

TABLE XI

REDUCTION OF DPN⁺ BY ADDITION OF SUCCINATE (90 SEC. AT 0°) TO RABBIT-HEART SARCSOMES

Unpublished experiment of M. Bailie (Eo71). Sucrose, 0.18 M; succinate, 40 mM. Values in μ moles/g. protein.

DPN ⁺ ,	before succinate	2.81
	after succinate	1.34
JDPN ⁺		-1.47
DPNH,	before succinate	1.57
	after succinate	4.24
JDPNH		2.67

In the course of these experiments, we have repeated Chance and Hollunger's [19] observation on the effect of adding succinate to sarcosomes in State 4 (i.e. deficient in ADP) in a medium already containing glutamate. Our results agree with those of these authors in that succinate brought about a considerable reduction of the DPN (Table X). Addition of succinate to the sarcosomal suspension for only 90 sec. at 0° (cf. Tables V and VI) also caused the appearance of much DPNH (Table XI).

Conclusion

Our results agree with those of Klingenberg and Slenczka [8] in that they fail to show the presence in most preparations of fresh rat-liver mitochondria of detectable amounts of the "extra DPN" of Purvis. We do, however, often find appreciable amounts of a compound with the properties of "extra DPN" after incubation of the mitochondria with substrate, in the absence of P_i . Rabbit-heart and pigeon-breast sarcosomes are a much more consistent source of "extra DPN".

Further work is required to establish the significance of the "extra DPN" and it would be premature to speculate on its nature. Concerning whether it is more likely to be a DPN^+ or a $DPNH$ compound, the reader is referred to a discussion between one of the writers and Dr. B. Chance, published in the *Proceedings of the International Symposium on Haem Compounds* (Canberra, 1959).

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Discussion

CHANCE: I should be glad to clarify an ambiguity which Prof. Slater pointed to; in the experiments published in the work with Hollunger, we had pretreated the guinea-pig kidney mitochondria with glutamate. Therefore some ATP was produced by the α -ketoglutarate step. The addition of succinate therefore produced a rapid and complete reduction of DPN. For the last 8 to 10 months we have been working with pigeon-heart preparations which, when freshly prepared or after a day's ageing, do have a ATP requirement for DPN reduction in the presence of succinate. They will respire slowly but the DPN will not be reduced unless you add ATP. If they are suspended in a medium containing phosphate, the respiration will increase and the DPN will slowly be reduced in several minutes. Thus this preparation is admirably suited to separate the substrate and energy requirements for the DPN reduction.

SLATER: I think there must still be some difference between our rabbit-heart sarcosome preparation and your pigeon-heart preparation because 90 sec. at 0° is probably a short time.

CHANCE: Yes; I think it is a question of endogenous substrate concentration.

Nucleotides and Mitochondrial Function: Influence of Adenosinetriphosphate on the Respiratory Chain

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It has been known since the discovery of oxidative phosphorylation that electron transport of the respiratory chain effects the phosphorylation of the adenine nucleotide system. The reverse control of electron transport by the phosphorylation of the adenine nucleotides has been demonstrated with the influence of ADP on respiration and on the redox state of nucleotides and cytochromes of the respiratory chain [cf. 1, 2, 3]. It could be shown only recently that the redox state of the respiratory chain can also be influenced by ATP in a reversal of the oxidative phosphorylation [4, 5, 6].

The relation between the ATP level and electron transport, as followed by the respiration and the redox state of the respiratory chain, is the subject of studies presented in this article. This aspect will be pursued both with respect to the intramitochondrial ATP as well as to the effect of external ATP. In this context we are concerned with the reversal of the oxidative phosphorylation, which was postulated to take place in the succinate and glycerolphosphate induced DPN reduction in mitochondria [7, 8, 9, 10]. Some related data on the major intramitochondrial nucleotide systems will be presented first.

Intramitochondrial nucleotide systems

The anion exchange chromatograms of Fig. 1 give an example of the pattern and behaviour of intramitochondrial nucleotides in two different functional states [11]. Only the pyridine and adenine nucleotide systems can be extracted in appreciable amounts from these muscle mitochondria. The concentration of reduced pyridine nucleotide in the perchloric acid extract of mitochondria is quantitatively determined by the concentration of the acid decay products of DPNH (ADP-ribose) and of TPNH (ADP-ribosephosphate) [12]. In confirmation of the results obtained by other methods, in the presence of succinate DPN is mostly reduced, whereas in the presence of dinitrophenol, DPN is oxidized. Similar but smaller changes are observed with the TPN system. As expected, in the presence

of succinate a higher ATP-level is formed than with dinitrophenol. However, it is remarkable that also in the presence of succinate appreciable amounts of ADP and AMP are found together with inorganic phosphate.

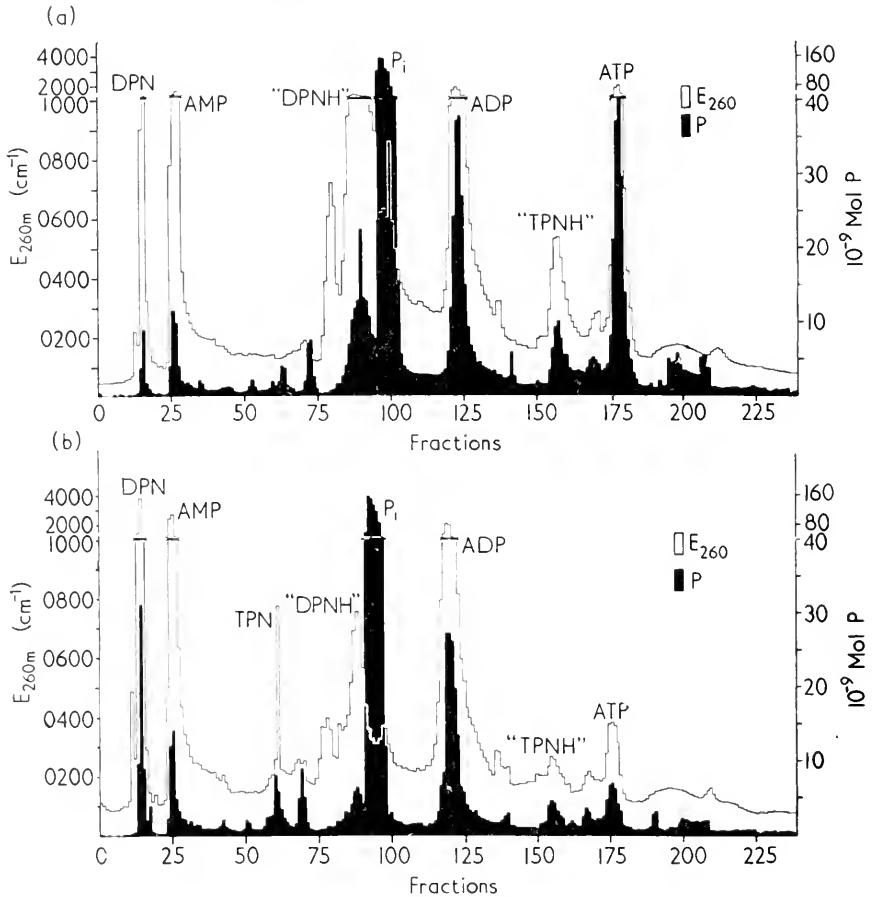


FIG. 1. Anion exchange chromatograms (Dowex 2) of perchloric acid extracts of pigeon-breast muscle mitochondria. Before extraction the mitochondria were incubated for 60 sec. in about 15 ml. of the reaction medium, containing 0.3 M sucrose, 10 mM triethanolamine-HCl-buffer, pH 7.2, 25°. This medium was oxygen (1 atm.) saturated.

Chromatogram a. Mitochondria (47 mg. protein) incubated under addition of 4 mM succinate.

Chromatogram b. Mitochondria (47 mg. protein) incubated under addition of 0.1 mM dinitrophenol.

In a first attempt to correlate functional states of the three major mitochondrial nucleotide systems, the phosphorylation state of the adenine

nucleotide system is compared with the redox states of the pyridine and flavin nucleotide systems under various conditions, as shown in Table I. These data have been obtained by enzymatic analysis. The mitochondria are saturated with substrate in the presence of oxygen and thus are in a condition facilitating the phosphorylation of the endogenous adenine nucleotides. The data refer to a steady state. The substrates can be divided into two groups: the DPN-specific substrates, pyruvate plus malate,

TABLE I
NUCLEOTIDE SYSTEMS IN MITOCHONDRIA

Comparison between the phosphorylation state of the adenosine phosphates and the redox state of DPN and flavoprotein.

Additions	$\frac{\text{ATP}}{\Sigma\text{AP}^*}$	$\frac{\text{DPNH}}{\Sigma\text{DPN}\dagger}$	$\text{FpH}_2\ddagger$ ($\mu\text{Mol. g. Prot.}$)
<i>Heart muscle</i>			
Succinate	0.56	0.75	0.30
Succinate, PO_4	0.54	0.70	0.24
Succinate, antimycin A	0.07	0.26	0.14
Pyruvate + malate	0.33	0.28	0.02
Pyruvate + malate, PO_4	0.62	0.37	0.03
<i>Flight muscle</i>			
Glycerol-1-P	0.45	0.55	0.51
Glycerol-1-P, PO_4	0.75	0.49	0.30
Glycerol-1-P, antimycin A	0.17	0.06	0.13
Pyruvate + malate	0.59	0.18	0.15
Pyruvate + malate, PO_4	0.73	0.04	0.08

* $\Sigma\text{AP} = \text{ATP} + \text{ADP} + \text{AMP}$.

† $\Sigma\text{DPN} = \text{DPN} + \text{DPNH}$.

‡ Calculated from extinction changes at 468–500 $m\mu$, using $\epsilon_{\text{mm}} = 8.5 \text{ cm}^{-1}$.

and the non-DPN-specific substrates, succinate and glycerol phosphate. The postulated energy supply for the succinate or glycerol-phosphate linked DPN reduction might be reflected in the intramitochondrial ATP-level. However, with both these groups of substrates about 50–70% of the adenine nucleotides are present as ATP. Addition of phosphate on top of the endogenous phosphate increases the phosphorylation, as is to be expected. In contrast, there are large differences between the two groups of substrates with respect to the degree of reduction of the DPN. With succinate or glycerol phosphate, DPN is reduced to a greater extent than with pyruvate plus malate. These results show that the redox state of the mitochondrial DPN appears not to be only a function of the endogenous energy supply, as expressed in the phosphorylation state of the adenine

nucleotide system, but also to be related to the reducing qualities of succinate or glycerol phosphate. This is supported by measurements of the absorption of mitochondrial suspensions at the flavin nucleotide wavelength, from which the amount of flavin nucleotide reduced has been estimated. With succinate or glycerolphosphate several times more flavin nucleotide is reduced than with pyruvate plus malate. Thus under these conditions of optimum intramitochondrial energy supply, a relation of the redox state between the pyridine nucleotide and the flavin nucleotide systems appears to exist.

When electron transport, and thus energy supply for oxidative phosphorylation, are inhibited by antimycin A, the adenine nucleotides are phosphorylated to a low degree only. It is to be noted that under these conditions the reduction of DPN and flavoprotein in the presence of succinate or glycerolphosphate is also diminished. This indicates that not only the reduction of DPN, but also of a part of flavoprotein is dependent on functioning oxidative phosphorylation. Again, the redox states of the DPN and flavin nucleotides behave in a parallel manner.

An energy-dependent reduction of flavoprotein had been observed and reported previously in flight muscle mitochondria [13, 14]. It was concluded that this flavoprotein cannot be on the main pathway of the oxidation of glycerolphosphate, since it was not reducible when electron transport was inhibited by anaerobiosis or antimycin A. Thus this flavoprotein may be reduced by the substrates through an energy-dependent hydrogen transfer in a way similar to the mitochondrial DPN.

Influence of exogenous ATP

EFFECT OF ATP ON PYRIDINE AND FLAVIN NUCLEOTIDES

In contrast to heart muscle and flight muscle mitochondria, the pyridine and flavin nucleotides in isolated skeletal muscle mitochondria remain oxidized if succinate or glycerolphosphate is added, although both substrates are active hydrogen donors for these mitochondria [4, 15]. Only the subsequent addition of ATP results in a large reduction of the DPN, as shown in Fig. 2. On addition of phosphate, DPN becomes largely re-oxidized. A similar ATP effect can be obtained in heart muscle mitochondria with another type of flavin specific substrate, in the presence of capronate. The extent of the DPN reduction is quantitatively measured by enzymic pyridine nucleotide analysis (Table II). DPN is further reduced to 45% by the addition of ATP in the presence of either glycerolphosphate or succinate. With the DPN specific substrates, pyruvate plus malate, a different picture emerges. In this case, DPN is reduced to about 32% by the substrates alone. ATP addition does not increase further the reduction of DPN.

These results show that added ATP can influence the redox state of intramitochondrial components of hydrogen transfer such as DPN and flavoprotein. It is to be assumed that ATP exerts its influence through back reactions of oxidative phosphorylation. The energy requirement for the DPN reduction in the presence of succinate or glycerolphosphate—so far only postulated on the basis of thermodynamic reasoning—has been directly demonstrated by these experiments. This phenomenon appears to

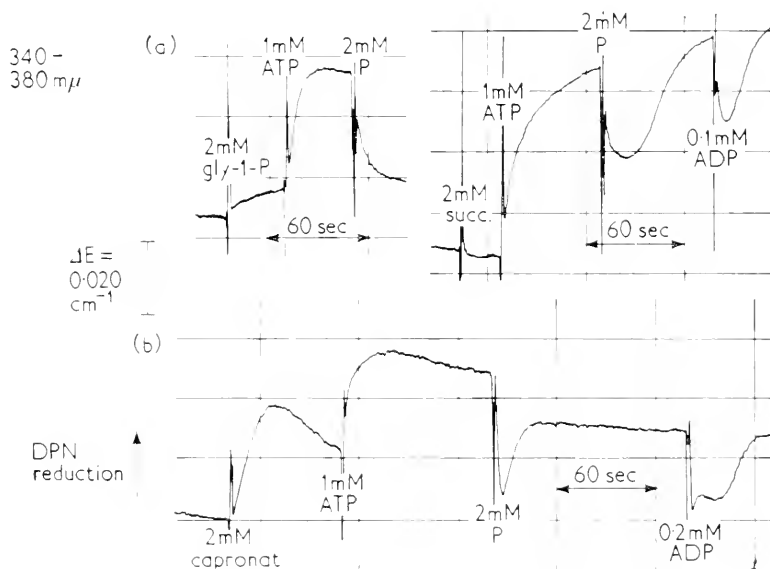


FIG. 2. The reducing effect of ATP on mitochondrial pyridine nucleotides in the presence of non-DPN-linked substrates. Double beam spectrophotometer recordings of suspensions of mitochondria. a. Skeletal muscle mitochondria, 2.8 mg. protein ml. b. Heart muscle mitochondria, 2.0 mg. protein ml. Incubated in 0.3 M sucrose, 1 mM EDTA, 10 mM triethanolamine-HCl-buffer, pH 7.2, 25, air-saturated.

apply to all substrates that can transfer hydrogen to the respiratory chain without a participation of DPN, since also with fatty acids DPN can be reduced at the expense of energy supply from ATP. In agreement with this picture no ATP is required for the reduction of DPN with DPN-specific substrates such as pyruvate plus malate.

Further work may be mentioned which was aimed at excluding other possible explanations of this ATP effect. Thus the specificity for ATP and the studies on the conditions for the ATP-effect furnished additional evidence that ATP acts in a reversal of oxidative phosphorylation [15].

In particular, the question arises why ATP is required for DPN reduction in isolated skeletal-muscle mitochondria in contrast to mitochondria

TABLE II

REDOX STATE OF PYRIDINE NUCLEOTIDES IN SKELETAL MUSCLE MITOCHONDRIA

Additions	DPNH $\mu\text{Mol/g. Prot.}$	TPNH $\mu\text{Mol/g. Prot.}$	$\frac{\text{DPNH}}{\Sigma\text{DPN}^*}$
Glycerol-1-P	0.72	0.47	0.17
Glycerol-1-P + ATP	2.05	0.58	0.47
Succinate	0.30	0.39	0.08
Succinate + ATP	1.85	0.60	0.44
Pyruvate + malate	1.80	0.55	0.32
Mitochondria after washing with serum albumin			
Glycerol-1-P	2.02	0.44	0.42
Succinate	3.01	0.46	0.63
Pyruvate + malate	2.28	0.44	0.48

Incubated in 0.3 M sucrose, 10 mM triethanolamine-HCl-buffer, 1 mM EDTA, pH 7.2, 25°. Concentration of substrates: 4 mM; ATP: 1 mM.

* $\Sigma\text{DPN} = \text{DPN} + \text{DPNH}$.

isolated from some other organs. The second part of Table II shows that, after washing the skeletal-muscle mitochondria with serum albumin, no ATP is required for DPN reduction. When added after the substrates, albumin is also effective in facilitating the DPN reduction, as shown in Fig. 3. It is assumed that albumin reverses an endogenous

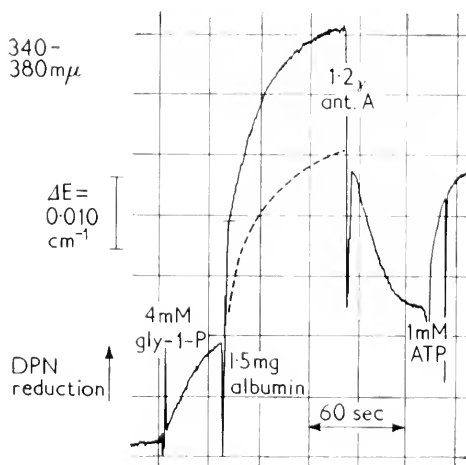


FIG. 3. The effect of serum albumin, antimycin A and ATP on the redox state of the mitochondrial DPN in the presence of glycerol-1-phosphate. The absorption trace (dashed curve) is corrected for the absorption due to albumin and by a shift of the pen position, for the absorption due to antimycin A (cf. legend Fig. 2.).

uncoupling of the mitochondria. In consequence, now the endogenous oxidative phosphorylation is more efficient in supplying energy for DPN reduction. No external ATP is required any more, since addition of ATP in this case would not increase the DPN reduction. As shown further in the experiment of Fig. 3, DPNH becomes oxidized when respiration, and thus oxidative phosphorylation, are inhibited by antimycin A. The energy for DPN reduction now has to be supplied by external ATP. The experiment shows, firstly, that for DPN reduction the oxidative phosphorylation system has not only to be intact, but also operative. Otherwise, ATP addition is required. Secondly, albumin acts only by protecting oxidative phosphorylation.

A similar reaction sequence can be observed with flavoprotein (Fig. 4). The partial oxidation of flavoprotein on addition of antimycin A, which

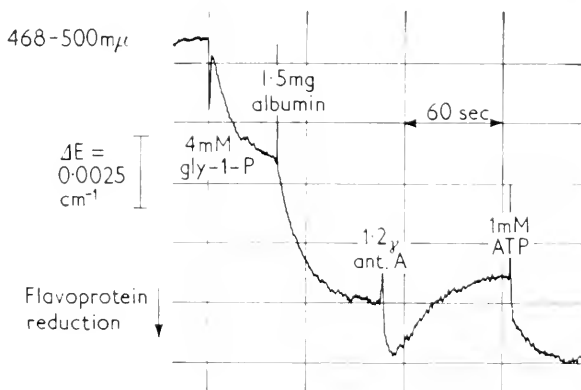


FIG. 4. The effect of serum albumin, antimycin A and ATP on flavoprotein in the presence of glycerol-1-phosphate (cf. legend Fig. 2.).

was previously mentioned (cf. Table I), demonstrates that the reduction of some flavoprotein depends on energy supply from oxidative phosphorylation. When inhibited, oxidative phosphorylation can be replaced by added ATP, which then effects a reduction of flavoprotein in the same way as with DPN.

EFFECT OF ATP ON CYTOCHROMES AND RESPIRATION

So far we have dealt with the influence of the ATP-level on the redox-state of DPN and flavoprotein. By these experiments an action of ATP on the DPN-flavin region only is conclusively demonstrated. That means that these experiments show primarily the reversal of the first phosphorylation step of the respiratory chain. It has now been possible to demonstrate also an influence of ATP on the redox state of the cytochrome region of

the respiratory chain and on the overall electron transport, i.e. the oxygen uptake, in the presence of succinate or glycerolphosphate (15). Both cytochrome *b* and flavoprotein (Fig. 5), in the presence of succinate or glycerolphos-

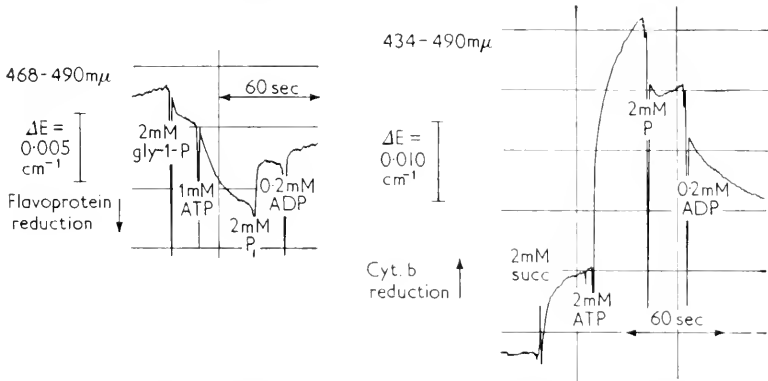


FIG. 5. The reducing effect of ATP on flavoprotein and cytochrome *b* in the presence of succinate or glycerol-1-phosphate (cf. legend Fig. 2).

phate are further reduced on the addition of ATP. The degree of reduction of cytochrome *b* increases from about 10% in the presence of substrate alone to 40% after the addition of ATP. Cytochrome *c*, as shown both with

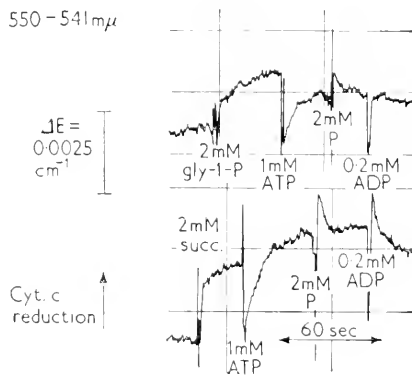


FIG. 6. The oxidizing effect of ATP on cytochrome *c* in the presence of glycerol-1-phosphate and succinate (cf. legend Fig. 2).

glycerolphosphate and succinate (Fig. 6) is however rapidly oxidized by addition of ATP. The degree of reduction of cytochrome *c* decreases from 12% to about 5 to 8%. Also cytochrome *a* is oxidized by addition of ATP (Fig. 7). In this experiment a low concentration of azide has been added in order to increase the reduction of cytochrome *a* and afterwards the

oxidizing effect of ATP, which otherwise is very small. Azide at this concentration does not yet inhibit noticeably the respiration (cf. also [16]). The reductive effect of ATP on cytochrome *b* and the oxidative effect of ATP on cytochromes *c* and *a* are reversed by addition of phosphate, ADP or dinitrophenol, as was found with the ATP-dependent reduction of DPN and flavoprotein.

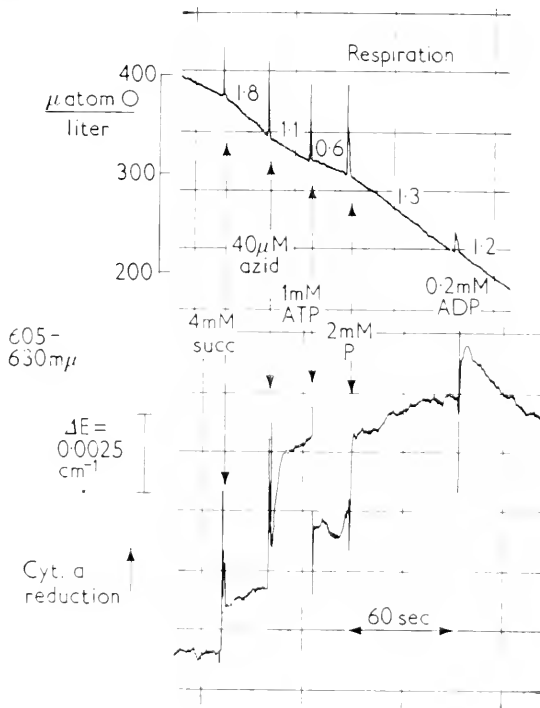


FIG. 7. The simultaneous inhibition of respiration and oxidation of cytochrome *a* in the presence of succinate on addition of ATP. Amperometric recording of oxygen consumption and simultaneous spectrophotometric recording at the α -band of cytochrome *a*.

The interaction of ATP with the respiratory chain causes a "crossover point" of the redox changes of the respiratory components between cytochrome *b* and cytochrome *c*. The "crossover point" can be interpreted to indicate the reaction step at which the electron transfer is inhibited by ATP. Thus, it is to be expected that in the overall electron transport also, i.e. in the oxygen uptake, an inhibitory effect of ATP can be observed. As shown in the upper recordings of Figs. 7 and 8, after addition of ATP, the respiration of skeletal-muscle mitochondria, both with glycerolphosphate and succinate, is inhibited to about 50%. After further addition of phosphate or dinitrophenol, respiration is again accelerated synchronously with

the increase of reduction of cytochrome *a*. It appears that in these mitochondria, which are partly uncoupled, the respiratory control is increased by ATP. The "crossover point" between cytochrome *b* and cytochrome *c* shows further that the respiration is inhibited by the action of ATP at this point. We conclude that by the "crossover point" of ATP action the reversal of oxidative phosphorylation also at this step of the respiratory chain is demonstrated. It should be noted that in studies of ADP action on the respiratory chain in liver mitochondria, by Chance and Williams, a "crossover point" between cytochrome *b* and *c* in the opposite sense had

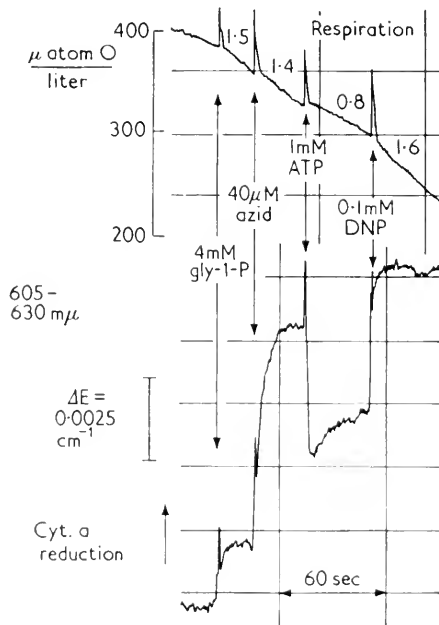


FIG. 8. The inhibition of respiration and oxidation of cytochrome *a* in the presence of glycerol-1-phosphate on addition of ATP (cf. legend of Fig. 2 and 7).

been observed [17], which led to assume a phosphorylation step at this point.

An inhibition of respiration with glycerolphosphate or succinate can also be obtained by albumin addition (Fig. 9). This inhibition is not abolished by phosphate but only by further addition of ADP. Thus albumin can also increase the respiratory control, in agreement with its assigned role of binding the endogenous uncoupling agents of mitochondria.

The kinetics of the redox changes initiated by ATP or albumin can also be explained on the basis of the proposed mechanism. The oxidation of cytochrome *c* or cytochrome *a* on addition of ATP is very rapid, followed

by a slow reduction; whereas the ATP-induced reduction of DPN or flavoprotein is comparatively slow. Also the respiration is inhibited immediately after the addition of ATP. Thus the rapid inhibition of the overall electron transport by ATP appears to be reflected in the cytochromes. This was to be expected since cytochromes *c* and *a* are thought to be in the direct electron transport of succinate or glycerolphosphate oxidation. On the other hand, the assumed reversal of electron transport

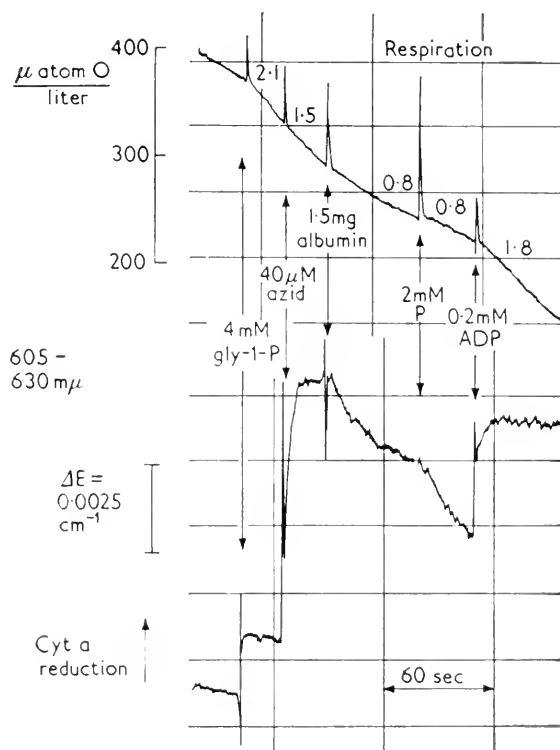


FIG. 9. The effect of albumin on respiration and on the redox state of cytochrome *a* in the presence of glycerol-1-phosphate.

can account for the lower velocity of the ATP-induced reduction of DPN and of flavoprotein. These components receive reducing equivalents in a reverse reaction from succinate of glycerolphosphate at a speed which may depend on the velocity of the energy supply. The parallel behaviour between cytochrome oxidation and the inhibition of respiration extends also to the effect of albumin (Fig. 9). Both the inhibition of respiration and initiation of cytochrome oxidation increase slowly after the addition of albumin. This kinetic behaviour is understandable on the grounds of the proposed mechanism of albumin action.

Conclusions

The experimental results of an influence of ATP on the respiratory chain are summarized as follows:

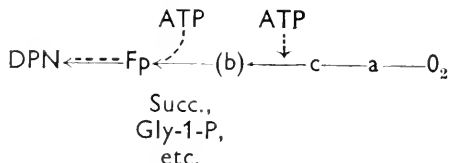
1. ATP can affect the reduction of DPN in the presence of flavin-specific substrates, such as succinate, etc. Thus the energy requirement of the DPN reduction in the presence of these substrates is directly demonstrated.

2. ATP can affect also the reduction of a flavoprotein, the reduction of which had been shown to depend on operative oxidative phosphorylation.

3. ATP can influence the overall electron transport by inducing respiratory control.

4. ATP addition causes a "crossover point" of redox changes at the respiratory chain between cytochrome *b* and *c*. cf. (15).

These results are interpreted as demonstrating the reversibility of oxidative phosphorylative reactions. At least two phosphorylation steps of the respiratory chain are shown to be reversible.



In the DPN-flavin region a complete reversal of the oxidative phosphorylation, including also a reversal of electron transfer, can be effected. In the cytochrome region the reversal of the reactions between ATP and the respiratory chain is seen. The interaction of ATP at the cytochrome level also controls the overall electron transport of succinate or glycerol-phosphate oxidation.

There are two aspects which should be briefly mentioned on the basis of these results. Firstly, the elucidation of the mechanism of oxidative phosphorylation depends greatly on the knowledge about the intermediates of the phosphate transfer reactions. The reversibility of oxidative phosphorylation presents in principle the possibility to estimate the energy content of the intermediates. Second is the physiological meaning of energy-dependent hydrogen transfer from flavin to pyridine nucleotide in mitochondria, as originally proposed by Krebs [18]. In this case, hydrogen from succinate or fatty acid oxidation would not be transferred to oxygen, generating ATP in oxidative phosphorylation, but, with expenditure of energy, to the DPN or TPN systems of the mitochondria. It appears possible to imitate such a system in experiments with liver mitochondria where hydrogen in the presence of ATP can be transferred from succinate to α -ketoglutarate with the formation of glutamate [19].

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Discussion

SLATER: May I take up the point we were discussing with Dr. Chance a moment ago. You require ATP for the reduction of succinate in your freshly prepared rat-skeletal-muscle sarcosomes, but when they are treated with albumin ATP is no longer required. You suggest that the albumin removes an uncoupler. Now Dr. Chance's pigeon-heart sarcosomes require ATP and our rabbit-heart sarcosomes do not require ATP. The difference between Dr. Chance's results and ours could then be explained by Dr. Klingenberg by the presence or absence of an uncoupler whereas Dr. Chance would prefer to explain it by the absence or presence of endogenous substrate. Is it possible, then, that albumin is removing an endogenous substrate which is a fatty acid rather than an uncoupler?

KLINGENBERG: I would say that albumin removes the uncoupler or may preserve endogenous substrate because ATP is no longer required.

CHANCE: I would be inclined to agree with Dr. Klingenberg's view that we either add or preserve the endogenous substrate so that energy would be available, however, for the reduction of the pyridine nucleotide on addition of α -glycerophosphate or succinate.

SLATER: I thought you said that albumin was removing an uncoupler.

KLINGENBERG: The general opinion is that it removes an uncoupler.

CHANCE: You can remove an uncoupler by ATP.

KLINGENBERG: I presume that skeletal muscle mitochondria are slightly uncoupled and also have in the presence of endogenous substrate a rather low level of ATP or high energy substances.

SLATER: Have you in mind as an uncoupler the unsaturated fatty acids?

KLINGENBERG: Possibly.

CHANCE: What is the effect of ATP when you have added glycerophosphate to the skeletal muscle but not phosphate or ADP?

KLINGENBERG: ADP cannot induce DPN reduction. The respiration is not stimulated.

CHANCE: We have been studying for some time the ADP-inhibition of succinate oxidation. When you add ATP to mitochondria which are not too tightly coupled, you may produce ADP and phosphate. Under these conditions I expect the same as you have observed, cytochrome *c* goes oxidized, and respiration may be inhibited. I think it is something which should be controlled in these preparations because they are sensitive to small amounts of ADP which will inhibit respiration strange as it may seem.

While I certainly agree that ATP can reverse electron transfer, I do not know whether you can do it in the presence of oxygen and thermodynamics speaks against it. I do not question the phenomenon but I am not sure whether you have demonstrated it to me at the cytochrome level.

Two comments occur to me: first, when the mitochondria became anaerobic, you report flavin slowly became oxidized. This may be hard to measure accurately. The second point is that we get much more DPN reduction on adding ATP in the presence of succinate, in fact there isn't just any more DPN to be reduced when mitochondria go anaerobic with added ATP, but you only get 40%. Is this a difference in preparations?

KLINGENBERG: This may be; we sometimes get 60% but never more; I do not think this is very significant. In the presence of ADP or phosphate, ATP does not inhibit respiration and does not oxidize cytochrome *c*. This would be an argument against the hypothesis that ADP is generated by the addition of ATP and thus has the effect on cytochrome *c*. Also in the presence of DNP no oxidation of cytochrome *c* or inhibition of respiration occurs on addition of ATP. I do not think that we have observed an electron transport reversal in the cytochrome region. I think we have observed a reversed interaction of ATP with the cytochromes which results in an increase of the respiratory control.

CHANCE: In the experiments I reported this morning, special precautions were taken to exclude the possibility that the electron acceptor for the observed oxidation of cytochrome *c* in the presence of ATP was oxygen. If it were oxygen, then obviously no "reversed interaction of ATP with the cytochromes" could have been proved. In Dr. Klingenberg's excellent experiments, which have just been reported, oxygen was present and no inhibitor of the oxidase was added; the system being in a steady state. Thus, an ATP induced inhibition of electron transfer would be sufficient to explain the observed results; there being no demonstration that the acceptor of electrons from cytochrome *c* was a substance at a lower and not at a higher redox potential the former would be required in reverse electron transfer.

KLINGENBERG: I had stated in my report that we interpret the oxidation of the cytochromes *c* and *a* to demonstrate an interaction of the ATP with the cytochromes by a reversal of oxidative phosphorylation. Although oxygen had not been excluded, these experiments should show the reverse interaction of ATP by way of oxidative phosphorylation reactions as clear as, in the opposite manner, an influence of ADP and phosphate on the redox state of the cytochromes shows an interaction by way of oxidative phosphorylation. This is further supported by the increase of the respiratory control after addition of ATP.

The Role of ATPase in Oxidative Phosphorylation*

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In previous communications we reported the resolution of mechanically fragmented beef heart mitochondria into a particulate and a soluble protein component, both of which were required for oxidative phosphorylation [1, 2]. The particulate fraction catalyzed the oxidation of a number of substrates with little or no concomitant phosphorylation. The addition of the soluble component to the particulate fraction recoupled the respiration to phosphorylation. A summary of the properties of the reconstituted system and of the soluble factor, as well as some of the more recent developments with this system will form the subject of this discussion. Since a detailed description of the experimental procedures was presented elsewhere [3, 4] only the salient features will be considered here.

Beef heart mitochondria, prepared according to the method of Green *et al.* [5], were disrupted *in vacuo* in the presence of glass beads by means of a high-speed reciprocal Nossal shaker [6]. The suspension was centrifuged for 20 min. at $26\,000 \times g$ yielding a brown, gelatinous residue which was discarded and a yellow, turbid supernatant fluid. The supernatant solution was recentrifuged at $105\,000 \times g$ for 30 min. A red-brown gelatinous residue (residue 1) and a faintly turbid, yellow supernatant fluid were obtained. The supernatant fluid was decanted and clarified by centrifugation for an additional 30 min. at $105\,000 \times g$, yielding a clear yellow solution. Residue 1 was washed by homogenizing in 0.25 M sucrose containing 0.002 M EDTA and centrifuged at $105\,000 \times g$ for 30 min. The washing procedure was repeated with 0.25 M sucrose and the final residue 1 was suspended in 0.25 M sucrose.

As shown in Table I residue 1 catalyzed the oxidation of succinate with little or no accompanying phosphorylation. Addition of increasing amounts of the supernatant solution ($105\,000 \times g$) resulted in almost a tenfold increase in phosphate uptake. The coupling factor had no significant effect

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on respiration and appears, therefore, to be primarily concerned in the phosphorylation mechanism. Phosphorylation in the reconstituted system is uncoupled by 2,4-dinitrophenol as well as by a number of other recognized uncouplers including dicoumarol, chlorpromazine and triiodo-L-thyronine. The maximal P:O ratios obtained in this particular experiment are somewhat less than those generally observed. The average maximal P:O ratio was 0.6, with a range of 0.4 to 0.8. During the early phases of this work, different preparations of the particulate fraction exhibited residual and variable phosphorylation activity. Nevertheless, the addition of the supernatant fraction never failed to result in a marked increase in

TABLE I

EFFECT OF THE SUPERNATANT SOLUTION ON PHOSPHORYLATION ACCOMPANYING SUCCINATE OXIDATION

Each Warburg vessel contained 0.05 M succinate, pH 7.4, 0.004 M MgCl₂, 0.002 M ATP, 0.012 M potassium phosphate buffer, pH 7.4, 0.032 M glucose, 0.005 M tris, pH 7.4, 0.001 M EDTA, 0.06 mg. yeast hexokinase (25 to 40 units/mg.), 1.6 mg. of the particulate fraction and the indicated amounts of the supernatant solution (105 000 × g) in a final volume of 0.5 ml. Where added, 2,4-dinitrophenol (DNP) was 0.0005 M. Incubations were carried out for 36 min. at 30°.

Supernatant solution	O ₂ uptake	P _i uptake	P:O
μg. protein	μatoms	μatoms	
None	5.6	0.3	0.05
75	5.0	0.8	0.16
150	4.7	1.8	0.38
300	4.9	2.2	0.45
2400	5.9	2.7	0.46
2400 + DNP	6.1	0.4	0.04

the P:O ratio. In subsequent work, disruption of the mitochondria was carried out in the presence of EDTA, which in confirmation of the results reported by Linnane [7], consistently yielded particles in which phosphorylation was either low or absent. For reconstitution, these particles were preincubated with the soluble protein and Mg⁺⁺ and an aliquot of the mixture was then added to a Warburg vessel containing the otherwise complete reaction mixture (cf. Table I).

Table II illustrates the dependency on the coupling factor for phosphorylation associated with the oxidation of various substrates. It may be seen that isocitrate was oxidized by the submitochondrial particles at about one-half the rate of succinate and that no esterification of P_i occurred in the absence of the purified coupling factor. The oxidation of β-hydroxybutyrate required the addition of DPN while glutamate oxidation occurred

only if the system was supplemented with both DPN and glutamic dehydrogenase. Again, both β -hydroxybutyrate and glutamate were oxidized without uptake of P_i unless the coupling factor was present. The maximal P:O ratio obtained was independent of the nature of the substrate undergoing oxidation, suggesting that only phosphorylation sites in the respiratory chain between succinate and oxygen contribute to the P:O ratio. A more precise localization of the phosphorylation site(s) is currently under investigation.

Purification and characterization of the coupling factor revealed the presence of a Mg^{++} -dependent, dinitrophenol-stimulated ATPase. The generally accepted concept that the mitochondrial ATPase is functionally

TABLE II

EFFECT OF THE PURIFIED COUPLING FACTOR ON PHOSPHORYLATION ASSOCIATED WITH THE OXIDATION OF VARIOUS SUBSTRATES

The experimental conditions have been previously described (cf. Table II [4]).

Substrate	Coupling factor	O ₂ uptake		P _i uptake		P:O
		μA min.	mg.	μA min.	mg.	
Succinate	—	0.25		0.00		0.00
	+	0.29		0.12		0.41
DL-isocitrate	—	0.14		0.00		0.00
	+	0.14		0.052		0.38
DL- β -hydroxybutyrate	—	0.054		0.003		0.05
	+	0.041		0.020		0.49
L-glutamate	—	0.040		0.003		0.07
	+	0.050		0.023		0.46

related to the enzymic mechanism of oxidative phosphorylation prompted us to examine the relationship between the ATPase and coupling activities. Since the most highly purified preparations which hydrolyzed 80 to 100 μ moles of ATP min. mg. protein induced phosphorylation coupled to oxidation in the presence of the particles, the question arose whether these activities were located in the same protein.

ATPase activity, which was measured in a system using phosphoenolpyruvate and pyruvate kinase as a regenerating system for ATP, was markedly higher in the presence than in the absence of the regenerating system. This is due, at least in part, to the fact that the pyruvate kinase system removes the ADP which is inhibitory to the enzyme. ATPase activity was increased 50 to 75% by the addition of 5×10^{-4} M 2,4-dinitrophenol.

Studies on the purified ATPase revealed properties consistent with its participation in coupled phosphorylation and similar to those described

for the particulate enzyme of mitochondria and phosphorylating mitochondrial fragments. Some of these properties are summarized in Table III. While a number of divalent cations including Co^{++} , Mn^{++} , Fe^{++}

TABLE III
PROPERTIES OF ATPASE

1. Divalent cation required for activity
2. Stimulated by 2,4-dinitrophenol
3. Hydrolyzes ATP, ITP, GTP, and UTP
4. Inhibited by ADP but not IDP
5. Stoichiometry: $\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{P}_i$
6. Exhibits "latent" activity phenomenon

and Ca^{++} substituted for Mg^{++} in activating the enzyme, only Mg^{++} and to a lesser extent Co^{++} gave rise to a dinitrophenol stimulation. The enzyme hydrolyzed ITP, GTP, and UTP in addition to ATP. However, it seems significant that only ATP hydrolysis was stimulated by dinitrophenol. Neither the nucleoside mono- nor diphosphates were hydrolyzed.

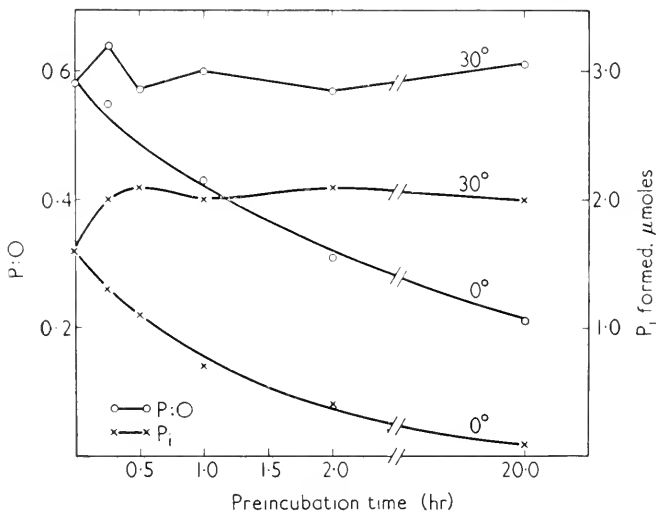


FIG. 1. Effect of preincubation temperature on ATPase and coupling activity. The purified enzyme was preincubated either at 0° or 30°. At the indicated time, aliquots were removed and the appropriate activity measured at 30° as described elsewhere [4].

The specificity of the ADP inhibition is of interest in view of the specificity of this nucleotide in oxidative phosphorylation. The "latent" activity phenomenon referred to in the table may actually be related to the well-known "latent" properties of mitochondrial ATPase [8-10]. It was

observed that incubation of the enzyme at 30° or in the presence of ATP at considerably higher temperatures resulted in an activation of the enzyme.

It was necessary, however, to accumulate more direct evidence that the ATPase and coupling activity were in fact catalytic expressions of a single protein. Compelling evidence in favour of this view was obtained by a number of procedures designed to selectively destroy one of the activities. Invariably these procedures resulted in a parallel destruction of both activities. The most striking of these parallelisms was noted during the later stages of the purification procedure when the ATPase activity became extremely unstable. Further investigations revealed that the purified preparation was markedly cold-labile. That is, the activity of the ATPase declined rapidly at ice bath temperature while at room temperature the activity generally increased. This rather unusual lability was also displayed by the coupling activity. These results are shown in Fig. 1. In

TABLE IV
PROTECTION BY ATP AGAINST HEAT INACTIVATION OF ATPASE AND
PHOSPHORYLATION ACTIVITY

A solution of the purified enzyme containing 1.6 mg. protein/ml. was divided into appropriate aliquots and heated for 4 min. under the indicated conditions. The ATP, when present, was 4×10^{-3} M. Assays for coupling activity (P:O) were carried out as described previously [4] with 0.038 mg. of the coupling enzyme and 0.495 mg. of the particulate fraction. In the absence of the coupling enzyme, the P:O ratio was 0.05. The ATPase assay was carried out in the presence of the ATP regenerating system [3].

Pretreatment	P:O	ATPase $\mu\text{moles P}_i/\text{mg.}/10'$
None	0.46	342.0
60°	0.16	59.0
60° + ATP	0.55	342.0

this experiment, aliquots of the purified protein fraction were preincubated either at 0° or 30° for the indicated intervals and then added to the appropriate assay system. Both assays were carried out at 30°. As may be seen, the rapid rates of inactivation of these two activities at 0° were strikingly parallel while at 30° both activities were retained for over 20 hr. The enzyme may be kept, however, at 4° as a suspension in 50% ammonium sulphate for 3 weeks without appreciable loss in either ATPase or coupling activity.

Exposure of the purified enzyme to elevated temperatures also failed to achieve a separation of the two activities. As shown in Table IV, both activities were inactivated at 60° to a similar extent and were completely protected by ATP.

Similarly, a 2-hr. dialysis at room temperature resulted in parallel losses of both activities. These results are presented in Table V. The addition of ATP to the dialyzing solution at a final concentration of 0.005 M again resulted in considerable protection of both activities. Various salts, for example, ammonium sulphate, ammonium chloride or potassium sulphate, added to the dialyzing medium at concentrations ten times higher than that of the ATP also prevented to a large extent the loss of both activities. Attempts to reactivate the dialyzed enzyme by the addition of boiled enzyme, cold inactivated enzyme or several known cofactors have been unsuccessful.

TABLE V

PROTECTION BY ATP AGAINST DIALYSIS INACTIVATION OF ATPase AND COUPLING ACTIVITY

2.8 mg. of the coupling enzyme were dissolved in 1.5 ml. sucrose-tris-EDTA and divided into three 0.5 ml. aliquots. Dialysis was carried out at room temperature for 2 hr. vs. 0.25 M sucrose-0.01 M tris, pH 7.4. ATP was 0.005 M when added. Assays for coupling activity (P:O) were carried out as described previously [4] with 0.038 mg. of the coupling factor and 0.620 mg. of the particulate fraction per vessel. In the absence of coupling factor the P:O was 0.03. ATPase activity was measured with the ATP regenerating system [3].

Dialyzing solution	P:O	ATPase $\mu\text{moles P}_i/\text{mg.}/10'$
None (undialyzed control)	0.48	360.0
Sucrose-tris	0.09	26.0
Sucrose-tris-ATP	0.33	172.0

Additional evidence for the single enzyme concept was obtained from an examination of the effect of uncouplers on the two activities. Some of these results are summarized in Table VI. In general it was found that all compounds which affect the ATPase (i.e. either inhibit or stimulate) also uncoupled oxidative phosphorylation. *p*-Chloromercuribenzoate, azide, Dicummarol, dihydrovitamin K₁ diphosphate (a water-soluble derivative of vitamin K₁) and triiodothyronine inhibited both the phosphorylation activity and the ATPase activity of the purified factor at concentrations between 5×10^{-4} and 5×10^{-6} M. Dinitrophenol and pentachlorophenol, two potent uncouplers of oxidative phosphorylation, inhibited the phosphorylation activity and stimulated the ATPase activity. Azide inhibited the ATPase activity to a greater extent in the absence than in the presence of dinitrophenol with the result that the stimulation by dinitrophenol was in effect increased from 50 to 300 or 400%. The opposite effect was observed with *p*-chloromercuribenzoate which completely eliminated the dinitrophenol stimulation.

Finally, in agreement with current concepts of the mechanism of oxidative phosphorylation which predict that the ATPase and $^{32}\text{P}_i$ -ATP exchange reactions are involved in a common reaction sequence in oxidative phosphorylation, it was found that the purified ATPase was required to reconstitute a dinitrophenol sensitive $^{32}\text{P}_i$ -ATP exchange. Representative data are presented in Table VII. Aliquots of a given particulate preparation were used to measure either the esterification of P_i in the presence of succinate or the incorporation of $^{32}\text{P}_i$ into ATP in the absence of added substrate. As may be seen, neither the particles nor the purified ATPase when tested alone catalyzed an appreciable $^{32}\text{P}_i$ -ATP exchange or phosphate esterification. However, the addition of increasing amounts of

TABLE VI

EFFECT OF VARIOUS COMPOUNDS ON ATPase AND PHOSPHORYLATION ACTIVITY

Compound	P:O	ATPase
2,4-dinitrophenol	Inhibition	Stimulation
Pentachlorophenol	"	"
<i>p</i> -Chloromercuribenzoate	"	Inhibition
Azide	"	"
Dicoumarol	"	"
Dihydrovitamin K ₁ diphosphate	"	"
Triiodo-L-thyronine	"	"
Amytal	—	None
Potassium cyanide	—	None
Potassium fluoride	None	None
Warfarin	None	None

the purified ATPase to the particles resulted in parallel increases in both of these activities. 2,4-dinitrophenol abolished both reactions.

Based on the evidence presented, as well as on other supporting data, we have concluded that the catalytic site or sites responsible for the hydrolysis of ATP and for the coupling activity reside on the same protein. It became necessary, however, to explain the observation that during the course of purification the ATPase was purified to a greater extent than the coupling activity. The apparent greater purification of the ATPase was actually not based on the removal of other protein impurities, but depended to a large extent on an absolute increase in total units. These results indicate an activation of a hydrolytic site or removal of an inhibitor rather than a physical separation of the two components.

We look upon the hydrolysis of ATP by the coupling factor as an aberrant activity which the enzyme has acquired following the disruption of the mitochondria. Bound to the structure of the undamaged mitochondria, the hydrolytic potentialities of this protein are largely masked,

and during oxidative phosphorylation the enzyme functions primarily as a transfer agent. A similar suggestion was made many years ago to explain the latent ATPase of intact mitochondria [8-10].

We feel that the most logical site for the action of this enzyme in oxidative phosphorylation would be the terminal transphosphorylation step. The possibility, therefore, of an ADP-ATP exchange catalyzed by this enzyme was explored. Numerous attempts under various experimental conditions have thus far been unsuccessful. However, these

TABLE VII

EFFECT OF THE COUPLING ENZYME ON OXIDATIVE PHOSPHORYLATION AND THE $^{32}\text{P}_i$ -ATP EXCHANGE REACTION

The particulate fraction was preincubated with the coupling enzyme and Mg^{++} as described previously [4]. Aliquots of the preincubation mixture containing 0.580 mg. of the particulate fraction and the indicated amount of the coupling enzyme were added to the Warburg vessel for the assay of oxidative phosphorylation or to test tubes for the measurement of the $^{32}\text{P}_i$ -ATP exchange reaction. Oxidative phosphorylation was measured at 30° for 30 min. with succinate as substrate [4]. The $^{32}\text{P}_i$ -ATP exchange reaction was measured as described elsewhere [4]. Each tube contained 0.016 M ATP, 0.016 M MgCl_2 , 0.01 M tris, pH 7.4, 0.04 M $^{32}\text{P}_i$ (1.2×10^4 c.p.m./ μmole), 0.001 M EDTA, pH 7.4 and the preincubated enzyme mixture in a final volume of 0.5 ml. When added, dinitrophenol was 5×10^{-4} M.

Coupling enzyme	O_2 uptake	P_i uptake	P:O	$^{32}\text{P}_i$ -ATP
$\mu\text{g.}$	μatoms	μmoles		c.p.m./ μmole ATP
0	6.3	0.1	0.02	90
5	6.2	0.1	0.02	190
10	5.5	1.0	0.18	440
20	5.5	1.5	0.27	740
40	5.5	2.0	0.36	1030
20 + DNP	5.5	0.1	0.02	50

failures are not considered decisive in view of the predominance of the hydrolytic activity exhibited by the purified enzyme. Since azide was found to inhibit the ATPase activity at concentrations which do not uncouple phosphorylation, attempts were made to demonstrate the ^{14}C -ADP-ATP exchange in the presence of this compound. It was anticipated that appropriate concentrations of azide might inhibit the hydrolytic activity without affecting the transfer activity. These experiments were also unsuccessful. We have recently isolated from the submitochondrial particles a substance which is a potent inhibitor of the ATPase and appears to have no effect on oxidative phosphorylation in the reconstituted system.

This material is heat-stable, precipitable by trichloroacetic acid and nondialyzable. The possibility that this substance may restore the ADP-ATP exchange by masking the hydrolytic site of the ATPase and thus convert it to a transfer enzyme is being explored.

Finally, we would like to present the results of some recent attempts to resolve further the submitochondrial particles. If the particulate fraction which is recoupled by the ATPase is further disintegrated by sonic oscillation, a new particle is obtained (residue 2) which requires both the supernatant fluid from which these particles were separated as well as the purified ATPase in order to restore phosphorylation. These results are

TABLE VIII

THE REQUIREMENT OF FACTOR 2 FOR OXIDATIVE PHOSPHORYLATION CATALYZED BY RESIDUE 2

Experimental conditions as described in the text and [4]. Factor 2 was added directly to the vessels.

Residue	Coupling Factor 1	Factor 2	JO	JP _i	P:O
$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	μatoms	μmoles	
1 (310)	None	None	6.2	0.4	0.06
1 (310)	148	None	5.9	3.2	0.55
2 (310)	None	None	5.3	0.4	0.08
„	148	None	6.1	0.4	0.07
„	296	None	6.4	0.4	0.06
„	148	212	5.8	3.7	0.64

presented in Table VIII. The top half of the table merely shows the response of the original particles to the purified ATPase or as it is referred to here, coupling factor 1. The lower portion of the table shows that both the ATPase and the supernatant solution obtained after sonic disintegration (factor 2) is required to restore oxidative phosphorylation. Factor 2 is heat-labile, non-dialyzable, is precipitated at pH 5.4 and exhibits no ATPase activity. It does not catalyze either the $^{32}\text{P}_i$ -ATP or the ADP-ATP exchange even when supplemented with the ATPase, but is required together with the ATPase for the restoration of a dinitrophenol sensitive $^{32}\text{P}_i$ -ATP exchange to the particles.

Little is known concerning the mechanism by which the ATPase is linked to the electron transport chain, nor is anything known of the enzymic function of factor 2. Nevertheless, the resolution of the enzymes of oxidative phosphorylation represents a necessary first step toward the ultimate goal of demonstrating the mechanisms of this complex process.

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Discussion

CONOVER: As the cold lability of the ATP-ase might suggest a lipoprotein of some sort, I was wondering if you have checked the lipid contents of the protein?

PULLMAN: No we haven't; until recently we haven't been able to obtain the amount of purified enzyme required for many of the physical and chemical determinations which we would like to carry out. We think we have now solved this problem and plan to examine this aspect of the problem in the near future.

CONOVER: Also I might add that I have tried to demonstrate ADP-ATP exchange in magnesium-stimulated ATP-ase as prepared by Kielley and Kielley, and we have run into similar difficulties in trying to find an exchange in this enzyme.

LARDY: It is very interesting that *p*-mercuribenzoate inhibits the DNP portion of the ATP-ase. This is a property which is shown in partly aged mitochondria. We have found several years ago that we could inhibit endogenous ATP-ase activity and in some experiments with these mitochondria it actually increased; it did, however, eliminate the DNP-stimulated portion.

PULLMAN: In a few experiments we too have observed that PCMB stimulated the magnesium activated portion of the activity. However, by far the most consistent effect of PCMB was to eliminate the DNP stimulation without affecting the Mg^{++} activation.

LEHNINGER: It seems to me that the factors which Dr. Pullman and I have been studying in our laboratories are coming closer and closer together. I think we also share the view that this coupling mechanism has its critical point where the water site is created, possibly by an inhibitor such as U factor. I would like to suggest, however, the possibility that his coupling factor or ATP-ase is a complex enzyme in the same sense as actomyosin is, and is composed of two or more pieces. I have been thinking myself that our C factor is similar to your ATP-ase and that it in turn consists of two components one which may be M, and of course it will take further work to clarify all these things. One point I wanted to ask you was when you have

this latency phenomenon and you develop your ATP-ase activity, is this preparation capable of restoring phosphorylation even after the latent ATP-ase has appeared?

PULLMAN: Yes, it always maintains its coupling activity. The only discrepancy with regard to the two activities is that during purification the ATP-ase is "activated" while the coupling activity is not.

PACKER: It is interesting that the nucleotide specificity for ATP-ase is also very similar to that for actomyosin, namely that the all four nucleotides catalyze the reaction, but only the ATP-ase is DNP-stimulated. These are similar to the properties of actomyosin B as reported by Blum and Morales.

PULLMAN: Yes, there is a remarkable parallelism between the mitochondrial ATP-ase and myosin ATP-ase. However, I think that in the case of myosin, the hydrolysis of some of the other triphosphonucleosides is also stimulated by DNP or is that what you said?

PACKER: I only wish to comment that there are remarkable similarities between this ATP-ase and actomyosin and it would appear that your enzyme may be a very promising choice as the mechano-protein which has received so much discussion this afternoon.

The Mechanism of Coenzyme Q Reduction in Heart Mitochondria

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Our laboratory and the Liverpool group have previously presented evidence [1, 2, 3] that Coenzyme Q (CoQ) is positioned between the flavoprotein and cytochrome c_1 in the mitochondrial succinoxidase system. Furthermore, it is generally accepted that cytochrome b , at least in non-phosphorylating particles, is not an obligatory electron carrier either in the reduction of CoQ or in its reoxidation by cytochrome c_1 [1]. However, CoQ does not react directly with the flavoprotein since the primary succinic flavoprotein isolated by the method of Singer *et al.* [4] does not catalyze the reduction of CoQ. We have isolated a soluble form of the succinic flavoprotein that can catalyze this reaction [5], and in this report we will present evidence that the non-haem iron associated with the succinic dehydrogenase functions as an electron carrier between the flavoprotein and the quinone.

TABLE I
SUCCINIC-CoQ REDUCTASE ACTIVITIES*

Preparation	μ moles CoQ reduced (min., mg. protein)
Beef heart mitochondria	1.1
Succinic-CoQ reductase [5]	56.0
Primary succinic flavoprotein [4]	0.0

* The succinic-CoQ reductase activities were measured by the method described in ref. [2].

Table I lists the succinic-CoQ reductase activities of heart mitochondria, the soluble succinic-CoQ reductase and the primary succinic flavoprotein. The succinic-CoQ reductase is about fifty times more active than the starting heart mitochondria while the primary succinic flavoprotein does not catalyze this reaction. It is apparent that the site necessary to link CoQ to the flavoprotein is still present in the Q reductase but is either lost or non-functional in the primary flavoprotein.

* The author is indebted to Dr. D. E. Green for his advice during the course of this work.

The turnover of the flavoprotein in CoQ reductase with CoQ as the electron acceptor is slightly faster than that of the same enzyme in the electron transport particle (ETP) with any of the electron acceptors listed in Table II. The calculated turnover of the succinic flavoprotein in ETP is based on the assumption that all the flavin in the particle released by acid only after tryptic digestion is part of the succinic dehydrogenase [4]. Either this assumption is not valid or some activation of the enzyme occurs during its isolation.

The soluble succinic-CoQ reductase contains 4.2 to 4.6 $m\mu$ moles flavin, 4.4 to 4.8 $m\mu$ moles haem, 34 to 38 $m\mu$ moles non-haem iron, and 0.18 to 0.20 mg. lipid per mg. protein. The ratio of flavin to protein in the

TABLE II
TURNOVER RATES* OF THE SUCCINIC FLAVOPROTEIN IN SOLUBLE AND PARTICULATE PREPARATIONS

Preparation	Flavoprotein concn. ($m\mu$ moles/mg. prot.)	Electron acceptor		
		CoQ	Phenazine methosulphate	O ₂
Primary succinic flavoprotein [4]	4.3	—	4100	—
Succinic CoQ reductase [5]	4.2	12 600	11 300	—
Electron transport particle [6]	0.19	10 000	9 700	10 100

* The turnover rates are expressed as moles of succinate oxidized per min. per mole of succinic flavin.

reductase is almost identical with that of the primary succinic flavoprotein and both forms of the dehydrogenase contain non-haem iron. Singer and his associates have reported that the ratio of iron to flavin in the primary flavoprotein is 4:1; whereas in the reductase the ratio is 8:1. About one-half of the non-haem iron can be removed from the reductase by prolonged aerobic dialysis against 10^{-3} M ethylenediamine tetraacetate, but the CoQ reductase activity of the enzyme is destroyed by this procedure.

The iron that is removed by aerobic dialysis is probably not adventitious iron since the enzyme can be dialyzed anaerobically for the same length of time without the loss of either iron or activity. Addition of ferric or ferrous ions to the enzyme after aerobic dialysis does not restore activity. It is possible that some functional group (i.e. thiol) required for iron binding is oxidized during prolonged aerobic dialysis.

In contrast to the primary flavoprotein the CoQ reductase contains haem and lipid. All the haem can be extracted with acid-acetone and its reduced pyridine haemochromogen is identical with that of protohaem. The spectrum of the dithionite reduced enzyme (Fig. 1), indicates that the haem prosthetic group is similar to the mitochondrial cytochrome *b*; however, not all the cytochrome *b* in mitochondria can be associated with the succinic flavoprotein since mitochondria contain about three times as much protohaem as succinic flavoprotein.

The cytochrome present in the CoQ reductase is not reduced by succinate (Fig. 1) so it cannot function as an electron carrier between the

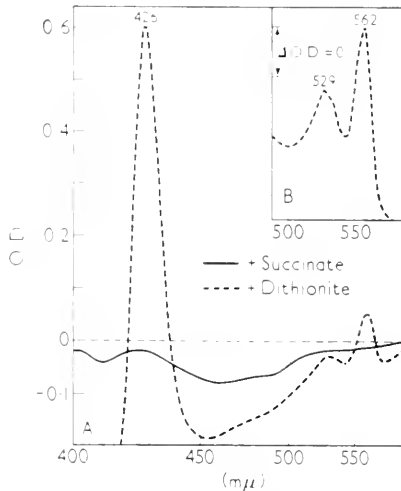


FIG. 1. Difference spectra of succinic-CoQ reductase (oxidized vs. reduced). The enzyme was dissolved in 0.1 M phosphate buffer pH 7.4 at a final concentration of 1.16 mg. protein per ml. The enzyme was first reduced with succinate (100 μ moles/ml.) and then with dithionite.

flavoprotein and CoQ. If, however, the enzyme is first reduced with limiting amounts of dithionite the cytochrome is reoxidized by fumarate (Fig. 2)—an observation which suggests that a functional link still exists between the flavin and haem groups. We have not been able to define the function of the cytochrome associated with the flavoprotein. One possibility suggested by the work of Conover and Ernster [14] is that cytochrome *b* is an intermediate electron carrier between extramitochondrial oxidative enzymes and the electron transport system.

Beinert and Sands have examined the succinic-CoQ reductase by electron paramagnetic resonance spectroscopy and they have reported that the enzyme contains a paramagnetic species that can be reduced by succinate and reoxidized by CoQ [7]. Since iron is the only transition metal

present in significant amounts in the isolated enzyme, we have determined, by a chemical method, the oxidation-reduction state of the enzyme bound non-haem iron in the isolated flavoproteins and a number of submitochondrial particles (Table III).

In agreement with the earlier work of Massey [8], we have found that the non-haem iron associated with the primary succinic flavoprotein is not reduced by succinate, but approximately 30% of the non-haem iron in the isolated Q reductase is reduced by substrate. Succinate, but not DPNH, reduces significant amounts of the iron in the succinic-cytochrome *c* reductase particle prepared by the method of Green and Burkhard [9]. This particle does not contain a functional DPNH chain and cannot

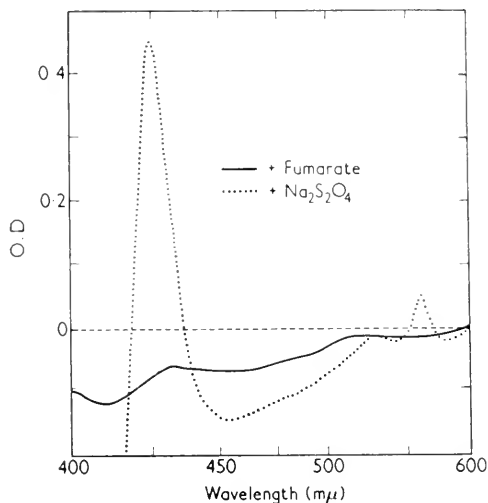


FIG. 2. Difference spectra (oxidized vs. reduced) of the succinic-CoQ reductase. The enzyme was first reduced with limiting amounts of dithionite and then fumarate (10 μ moles/ml.) was added.

catalyze the reduction of CoQ by DPNH; whereas, in the DPNH cytochrome *c* reductase particle [10] which is essentially free from the succinic flavoprotein, only DPNH reduces significant amounts of the non-haem iron. Either substrate can reduce approximately the same amount of the non-haem iron in ETP where both the DPNH and succinate electron transport chains are intact. All the iron potentially reducible in these preparations is reduced in a few seconds at 5°. Our studies on the rates of iron reduction, indicate that at 5° the non-haem iron is reduced as rapidly as CoQ.

Under the conditions given in Table III, the reduction of enzyme-bound iron is strictly substrate-dependent. The possible non-specific

reduction of iron by known redox components of the electron transport chain after the enzyme is denatured by ethanol can be excluded in a number of ways. Since in ETP the same amount of iron is reduced either in the presence of antimycin or anaerobically, it follows that cytochromes *c*₁, *c* and *a* cannot participate in this reduction. The succinic-CoQ reductase does not contain CoQ, and the cytochrome *b* present is not reduced; therefore, neither of these components reduces the non-haem iron during its extraction from the denatured protein. The possible interference of the

TABLE III
REDUCTION OF NON-HAEM IRON IN MITOCHONDRIAL SUBFRACTIONS*

Preparation	Total non-haem Fe	Percentage of total non-haem Fe reduced	
		by DPNH	by succinate
Primary succinic flavoprotein [4]	17	0	0
Succinic-CoQ reductase [5]	34	0	23
Succinic-cytochrome <i>c</i> reductase [9]	22	< 1	23
DPNH-cytochrome <i>c</i> reductase [10]	15	30	< 2
ETP _H [11]	9	39	30

* The procedure for measuring the redox state of enzyme-bound non-haem iron will be described in detail elsewhere [12]. A brief summary of the method is as follows: (i) The preparation is treated with KCN or antimycin A to block oxidation; (ii) substrate is added at zero time; (iii) the reaction is stopped by adding CdCl₂; (iv) the reduced non-haem iron is extracted with a mixture of ethanol (70%), *o*-chloromercuriphenol (5 mg. ml.), sodium acetate (100 μmoles ml., pH 4.6), and bathophenanthroline (0.1 mg. ml.); (v) the ferro-bathophenanthroline colour is measured at 535 mμ against a control to which substrate was added after the CdCl₂.

The cadmium ions [13] and the organic mercurial are necessary to prevent the non-enzymic reduction of iron by thiols.

flavoproteins cannot be entirely eliminated, but in most preparations the amount of iron reduced is considerably in excess of the flavoproteins and the non-haem iron is reduced in the succinic-CoQ reductase but not in the primary succinic flavoprotein. The ratio of flavin to protein in the latter two preparations is identical, which also indicates that non-enzymic reduction of the iron by reduced flavoprotein does not occur.

Amytal, a specific inhibitor of DPNH oxidase, blocks the reduction of non-haem iron by DPNH (Table IV), but malonate increases the total amount of iron reduced by this substrate. The reverse is true with succinate as substrate. Malonate blocks reduction of iron by succinate in ETP, but amytal increases the amount of iron reduced by succinate. In the presence of both succinate and DPNH the total amount of iron

reduced in ETP is the sum of the amounts obtained with either substrate alone. We do not have an explanation for these phenomena but the results demonstrate that the non-haem iron compounds are closely associated with the flavoproteins in the electron transport system, since all of the components after iron in the electron transport sequence can be completely reduced by either substrate.

TABLE IV

THE EFFECT OF INHIBITORS ON THE REDUCTION OF NON-HAEM IRON IN ETP

Inhibitor	Concentration	Percentage of total iron reduced by DPNH	Percentage of total iron reduced by succinate
None	—	39	3
Amytal	1×10^{-3} M	0	59
Malonate	2×10^{-2} M	65	0

The compound 2-thenoyltrifluoroacetone which chelates with iron is a highly efficient inhibitor of succinic-CoQ reductase activity (Table V). The level of the inhibitor required to block the reduction of CoQ by the reductase has only a small effect on the reduction of phenazine methosulphate and does not affect, at all, the phenazine reductase activity of the

TABLE V

INHIBITION OF SUCCINIC-COQ REDUCTASE ACTIVITY BY 2-THENOYL-TRIFLUOROACETONE*

Preparation	Electron acceptor (percentage inhibition)	
	CoQ	Phenazine methosulphate
Primary succinic flavoprotein [4]	—	0.0
Succinic-CoQ reductase [5]	98	17.0
ETP _{II} [11]	97	18.0

* The final concentration of the inhibitor— 1×10^{-4} M.

primary succinic flavoprotein. These data demonstrate that this metal chelating compound blocks a site required to link CoQ to the flavoprotein. The reagent does not combine with the flavin since it does not affect the phenazine methosulphate reductase activity of the primary succinic flavoprotein. Thenoyltrifluoroacetone does not remove non-haem iron but appears to form a strong complex with the enzyme-bound iron, which can no longer undergo oxidation and reduction. These data are consistent with

the assumption that the non-haem iron is an intermediary in electron flow between flavoprotein and CoQ but not between flavoprotein and phenazine methosulphate.

Spectra of the purified CoQ reductase suggest that the preparation contains, in addition to the flavin and haem prosthetic groups, some other component that can undergo oxidation and reduction. In addition to the flavin band at $450\text{ m}\mu$, succinate also reduces a component at approximately $415\text{ m}\mu$ (cf. the difference spectra shown in Fig. 1). The change in the spectrum upon reduction of the enzyme by succinate cannot be entirely due to the flavin even in the $450\text{--}460\text{ m}\mu$ region of the spectrum.

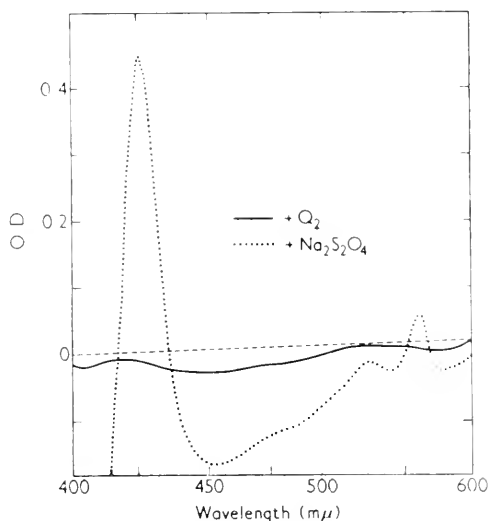


FIG. 3. These spectra were obtained under the same conditions as those of Fig. 2 except that CoQ_2 ($0.05\text{ }\mu\text{mole}$) was added instead of fumarate.

Even if all of the flavin is reduced, which is very unlikely, the decrease in optical density at $450\text{--}460\text{ m}\mu$ is considerably greater than could be attributed to the flavin alone.

The band at $415\text{ m}\mu$ is not contributed by flavin, since it is not re-oxidized by fumarate (Fig. 2). All the haem is reoxidized and essentially all the flavin should be reoxidized by fumarate. It is unlikely that the bands remaining after the addition of fumarate can be attributed to the flavin prosthetic group. In addition to the main band at $415\text{ m}\mu$ a broad band persists at $450\text{ m}\mu$, which again suggests that not all the reduction observed in this region is due to the flavin.

The components of the enzyme that remain reduced after the re-oxidation of the flavin and haem by fumarate are, however, reoxidized by

CoQ (Fig. 3). These data again demonstrate that the succinic-CoQ reductase contains a component other than the haem group that can function as an electron carrier between the flavoprotein and CoQ. Since non-haem iron is the only other known compound present in the enzyme that undergoes oxidation and reduction it is probable that the iron is responsible for the 415 $m\mu$ band observed in the reduced enzyme.

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Discussion

REDFEARN: This hypothesis raises the problem of the transfer of the electrons from flavoprotein which carries two electrons through a one electron carrying system (the iron) then again to a two-electron carrier. It was nice to think that the quinone could function as a semi-quinone and thus mediate the reaction between two-electron carriers and the one electron carrying cytochromes. Secondly, I noticed that you used Q_2 in your assay system. According to Crane's results on acetone-extracted preparations Q_2 produced an antimycin-insensitive pathway, which suggests that the site of action of Q_2 was not the same as that of the naturally occurring Q_{10} . Would you care to comment on this?

ZIEGLER: Is it necessary to assume that the flavoprotein is fully reduced during active electron transport? I would be more inclined to believe that in the intact succinoxidase particle the flavoprotein may be reduced only to the semiquinoid form, and if this is the case you would have one electron transfer during the oxidation of succinate.

With reference to the last point you raised, the reoxidation of reduced Q_2 is partly antimycin-insensitive and as you increase the length of the side chain you induce full antimycin sensitivity. However, in the reduction of Q the succinic-Q-reductase will react rapidly with either Q_{10} or homologues of Q and we have used

Q_2 in some of this work because it is far more soluble than Q_{10} , and in this way we can eliminate the necessity of adding the extra phospholipids required to solubilize Q_{10} , but we can always replace the Q_2 with Q_{10} plus phospholipids, and we have not detected a difference in specificity in the reduction of Q_2 or Q_{10} by succinate.

CHANCE: I was struck by the small degree of reduction of the iron in the various preparations in spite of the fact that they are inhibited by cyanide or antimycin A. Under these conditions I believe all the flavin would be reduced, and, if they contain Q, that would be similarly reduced. So do you have an explanation of values of only 30 to 60% reduction of the iron?

ZIEGLER: Some data which I did not show indicate that if you add both substrates to an ETP, which contains the two intact chains, the total amount of iron-reduced summates. DPNH reduces between 25 and 45% of the iron and succinate reduces about 30%; if you add both substrates 60 to 70% of the iron is reduced.

CHANCE: This is however considerably smaller than the percentage reduction of Q, and thus suggests that not all the non-haem iron is active in the pathway you are considering.

ZIEGLER: Yes, I would agree that not all of the iron functions in this capacity.

CHANCE: How many atoms of iron per molecule of Q are there?

ZIEGLER: In ETP there is twice as much non-haem iron as Q. In other words, per electron equivalent they are almost equal.

CHANCE: I am still not clear how many irons per Q are in the electron transfer pathway.

ZIEGLER: This would be a very difficult question to answer at this time since we do not know how much of the endogenous Q is involved in the oxidation of either succinate or DPNH. A more pertinent question would be, how many irons per flavoprotein are involved in the pathway to Q, and in all of our preparations capable of reducing Q at least 2 moles of iron per flavin undergo oxidation and reduction. In ETP the ratio of reducible iron to flavin is much greater than 2 and with both succinate and DPNH present the ratio can be as high as 15.

ESTABROOK: On the same point, I was wondering whether you have an explanation for your table (p. 258) of inhibitors where you show that when you used succinate as a substrate in the presence of amytal, which is an inhibitor of DPNH oxidation, you get the summation of iron reduced. In the same way as in the presence of malonate with DPNH as a substrate, you also find this summation.

ZIEGLER: I have no adequate explanation for these phenomena.

SINGER: Your slide shows eight atoms of iron per mole of flavin but, if I am not mistaken, your publication on the highly purified enzyme showed the same ratio as in the flavoprotein itself, that is four to one, and I am wondering what has happened in between to change these analytical data and whether this might throw some light on Dr. Chance's question.

We have been studying for some years the transformations that occur during the extraction of succinic dehydrogenase from the respiratory chain preparations. Since our results are relevant to the function of the metal in this enzyme, I might sum up the salient points. There are three main differences in the behaviour of succinic dehydrogenase between particulate (respiratory chain-bound) preparations and soluble ones. One is that the dehydrogenase is cyanide-sensitive in

respiratory chain preparations but not in the extracted, soluble form; second, that it has two reaction sites for phenazine methosulphate in particulate preparations but only one in soluble ones and thus in the particulate form the enzyme has twice the QO_2 in the phenazine assay that it has in soluble preparations; and, third, that in particulate preparations, but not in purified, soluble ones, it reacts with methylene blue, brilliant cresyl blue, and related dyes. In regard to all three criteria the CoQ-reductase of Ziegler and colleagues behaves like a respiratory chain preparation, as expected from the fact that the enzyme is still linked to cytochrome *b* in this particle. Work in our laboratory suggests that those properties of succinic dehydrogenase which are lost on solubilization are not fundamental characteristics of the flavoprotein itself but may be the consequence of the binding of some of the non-haem iron of the flavoprotein to the respiratory chain. We proposed some years ago that at least two of the four iron atoms of the isolated enzyme may act as ligands of the flavoprotein to the respiratory chain in particulate preparations. It is established that at least two of the four irons in the isolated flavoprotein do not function in oxido-reduction in purified preparations, although they might do so when bound in a particle. If so, they might also be involved in the catalytic cycling of CoQ in such particulate preparations as the CoQ reductase.

The anomalous absorption changes in the flavin region which Dr. Ziegler has observed, are, probably not fortuitously, very similar to those which occur in α -glycerophosphate dehydrogenase, a flavoprotein rich in iron, in the succinic dehydrogenase of *Micrococcus lactilyticus*, where iron has been shown to undergo oxido-reduction by the substrate, and in a rat liver enzyme which oxidizes inositol and which doesn't even have flavin but is an iron enzyme. These considerations would again suggest that part of the iron complement of the flavoprotein might undergo oxido-reduction in respiratory chain preparations.

ZIEGLER: The properties of the soluble succinic CoQ-reductase appear to be identical with those of the particle-bound dehydrogenase; the phenazine methosulphate reductase activity of the isolated enzyme is partly sensitive to cyanide. Cyanide also blocks the reduction of Q. We have tested a number of compounds that have been used to inhibit the particle-bound dehydrogenase, and disulphide compounds such as lipoic acid are very effective inhibitors of Q reduction.

The discrepancy between the concentration of iron in the enzyme reported here and in our earlier publication is due to a change in the method of estimating enzyme-bound iron. The ratio of 4 irons per flavin was obtained on a preparation that had been thoroughly dialyzed against a versene solution. However, the activity of the enzyme is destroyed by prolonged dialysis. Currently we remove extraneous iron by passing the enzyme through a column of Dowex A-1 chelating resin. This procedure does not destroy the Q-reductase activity of the enzyme and the ratio of non-haem iron to flavin is consistently 8:1.

ERNSTER: I would like to hear how you visualize the relationship of this mechanism to that prevailing in phosphorylating preparations, especially with respect to the participation of cytochrome *b*.

ZIEGLER: I have discussed this previously with Dr. Ernster and I think we are in full agreement. Could he put the mechanism we discussed on the board?

ERNSTER: Well, all I meant to ask is this: is this form of succinic dehydrogenase, which is now a Q-reductase, a cytochrome *b* reductase as well?

ZIEGLER: No, the cytochrome bound to the enzyme is, of course, not reduced and the enzyme will not catalyze the reduction of a number purified cytochrome *b*'s we have tested.

HOLTON: Could I ask you whether your conclusion that the succinate-Q reductase does not reduce cytochrome *b* is based on a difference spectrum of the reductase in the presence and absence of succinate?

ZIEGLER: This is one piece of evidence, yes.

HOLTON: Is it not just possible that your isolated reductase has cytochrome *b* present in the reduced form already without there being succinate present, and that is why the only change you show on addition of succinate is the reduction of Q. It seems to me to be very odd that succinate does not reduce cytochrome *b* in the presence of an enzyme which catalyzes the oxidation of cytochrome *b* by added fumarate.

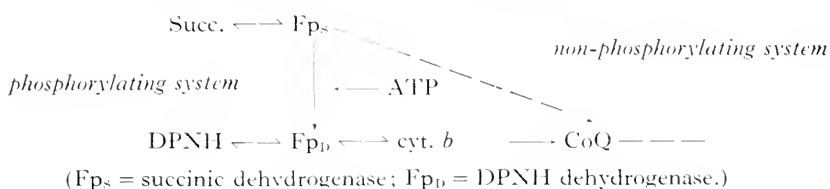
ZIEGLER: No. This is not a possibility. Most of the spectra we have of this enzyme are direct spectra and in no instance have we been able to keep the haem in the reduced form. Cytochrome *b* is quite auto-oxidizable.

CHANCE: This cytochrome *b* which comes along with the succinate-Q reductase has an absorption band at an appreciably different wavelength from the cytochrome *b* in the particle. I think it is very close to what Dr. Holton and I call "inactive" cytochrome *b*, because it is reduced only by dithionite, so I think it is a little premature to say that the properties of this kind of cytochrome *b* identify it with a particular pathway of electron transfer or phosphorylation.

HOLTON: Its reoxidation by fumarate indicates that this cytochrome *b* is in direct connection with the succinate-fumarate system.

CHANCE: But it may be by a different pathway.

ERNSTER: The mechanism we have been thinking about in connection with this activation of succinic oxidase in phosphorylating systems is this:



ZIEGLER: I agree with Dr. Ernster that this is one possibility we have to consider. However, as Dr. Chance pointed out, the haem attached to the enzyme may have been modified during isolation of the enzyme since all of the bands have been shifted to slightly lower wavelengths.

SLATER: Is this cytochrome *b* reduced by succinate in the presence of antimycin?

ZIEGLER: No, it is not.

SINGER: Much is made in discussions of this type of the reduction of flavin as measured at 450 or 460 mμ with or without a reference label. Perhaps I am merely voicing Prof. Keilin's recent caution in stating that the reduction of flavin in succinic dehydrogenase, etc., shuttles between the oxidized and reduced forms in its normal catalytic action. Since the isolated dehydrogenase does not undergo anything like a full bleaching even after activation by succinate and since its rate

of bleaching is not commensurate with the flavin undergoing such a complete cycle, although per mole of flavin the turnover number of the enzyme is exactly the same as in intact phosphorylating mitochondria, I think we must entertain the possibility that the enzyme shuttles between oxidized form and semiquinone and not between the oxidized and the reduced form which make all such measurements highly dubious. I think we should bear in mind that measurements at $450\text{ m}\mu$ do not indicate participation of flavoprotein in the respiratory chain.

ZIEGLER: I agree with Dr. Singer that a considerable amount of reduction we observe in the $450\text{ m}\mu$ region is not due to the flavin.

ESTABROOK: A few years ago you reported a very powerful inhibition by propionyl-CoA indicating that it acts in the flavin region of the respiratory chain. Is the iron reduced or not reduced, is the Q reduced or not reduced by propionyl-CoA?

ZIEGLER: We have not studied the effect of propionyl-CoA on non-haem iron reduction.

Reactions Involved in Oxidative Phosphorylation as Disclosed by Studies with Antibiotics

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Despite the great progress that has been made in understanding the process of oxidative phosphorylation, the number of reactions involved and the identity of all but a few of the reaction components remain unknown. Most of the information extant has been gained from studies with intact mitochondria. Ultimately the process must be examined in terms of the individual enzymes involved and the reactions they catalyze. But while isolation is in progress, new approaches to experiments with intact mitochondria may tell us how many components to look for.

To this end we have examined nearly one hundred highly toxic antibiotics for possible effects on respiration and phosphate transfer by mammalian mitochondria. Approximately 10% of the compounds tested have interesting effects on these processes (cf. [1]).

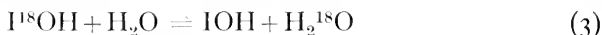
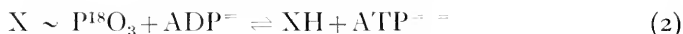
Two of the antibiotics—oligomycin and aurovertin—at concentrations of less than 1 μ g. per ml. strongly inhibit mitochondrial oxidation of all pyridine nucleotide-linked substrates. The inhibition is reversed by 2,4-dinitrophenol (DNP) indicating that these two antibiotics block enzymes involved in the energy-coupling mechanism, and have no effect on the respiratory enzymes.

Oligomycin was found [1] to inhibit the mitochondrial ATPase activity induced by either DNP, thyroid hormones, deoxycholate or Ca^{++} . Since dinitrophenol overcame the effect of oligomycin on respiration, and oligomycin nullified the effect of dinitrophenol on ATPase, it seemed possible that these two agents acted on the same enzymic site as competitors. However, a direct test of this hypothesis demonstrated that oligomycin did not act competitively in overcoming the effect of dinitrophenol [2].

Aurovertin differs from oligomycin in its effect on ATPase (Fig. 1). It depresses, but does not completely inhibit, the ATPase activity induced by DNP, Ca^{++} , or TCAP. It has no effect on the ATPase induced by Valinomycin, Triac, O-Me-Triac, DCA or ageing. Oligomycin overcomes all these (Fig. 1).

Both oligomycin [1] and aurovertin strongly inhibit the exchange of $^{32}\text{P}_i$ with the phosphate of ATP and the exchange of ^{18}O between $\text{P}^{18}\text{O}_4\text{H}^-$ and water.

For purposes of discussion, these experimental results may be examined in the context of the accepted, but hypothetical, reactions involved in fixing and transferring phosphate.



$\text{X} \sim \text{I}$ represents a product whose formation required the energy available from an oxidation-reduction reaction. Reactions 1+3 account

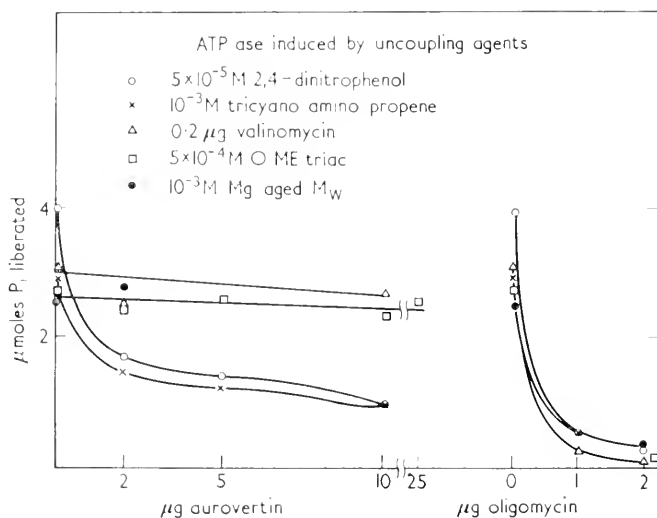


FIG. 1.

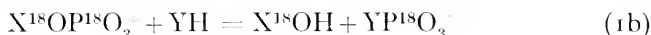
for the exchange of ^{18}O between phosphate and water [3]; reaction 2 accounts for exchange between ADP and ATP [4]. Reactions 1+2 account for $^{32}\text{P}_i$ -ATP exchange [5]. Reaction 3 is assumed to be spontaneous.

If we assume that reaction 1 is blocked by oligomycin and aurovertin, we learn that DNP must act prior to the stage at which P_i enters the sequence. If DNP prevents formation of $\text{X} \sim \text{I}$ or catalyzes the hydrolysis of $\text{X} \sim \text{I}$ or some earlier intermediate, it would prevent the inhibition of respiration by oligomycin and aurovertin. Likewise these antibiotics would block the effect of DNP on ATP hydrolysis.

But this scheme does not adequately explain the different effects of

these two antibiotics on ATPase induced by thyroid hormones, by valinomycin or by ageing (Fig. 1).

One manner of explaining the data would be to assume that two reactions are involved in the ^{18}O exchange reaction.



If oligomycin blocked reaction (1b) and aurovertin blocked (1a), each would block the effect of DNP on ATPase since DNP acts above reaction (1a). We are then led to the conclusion that thyroid hormones, valinomycin and ageing bring about ATP hydrolysis by catalyzing the hydrolysis of XOPO_3^- . Their effect would thus be blocked by oligomycin but not by aurovertin.

There are some data which detract from the appeal of this scheme. For example, valinomycin reverses the inhibition of mitochondrial oxidation by aurovertin. But perhaps some uncoupling agents act at both the DNP site and on XOPO_3^- . We are now making a more detailed comparison between aurovertin and oligomycin to determine whether they act at two different sites or whether there is some other explanation for the differential effect of these antibiotics on various ATPase activities of mitochondria.

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Discussion

LEHNINGER: These are very interesting results. In the ^{18}O exchange experiments on dignitonin preparations we recently reported [Chan, Lehninger, and Enns, *J. biol. Chem.* **235**, 1790 (1960)] that our reaction scheme for oxidative phosphorylation could not explain the higher incorporation of ^{18}O from H_2^{18}O into ATP than we were getting in the inorganic phosphate. The 2-stage mechanism Dr. Lardy suggests might offer some possibility of explaining this ^{18}O exchange, which otherwise can be explained only on a compartmentation basis.

ESTABROOK: Is the arsenate stimulation of oxidation inhibited by oligomycin as well as by aurovertin?

LARDY: We obtained aurovertin very recently. Of a large number of ATP-ase stimulations which we have tested including arsenate all are inhibited by oligomycin but we haven't tested them all with aurovertin. In addition to arsenate, we haven't tested dicoumarol on aurovertin yet.

HOLLUNGER: In a study of the effect of guanidine on oxidative phosphorylation [*Acta Pharmacol. et Toxicol.* **11**, Suppl. 1 (1955)] I came to the conclusion that guanidine decreased the respiration of mitochondria by inhibiting reactions connecting electron transport and ATP-generation. As Dr. Lardy now suggests the same point of attack for oligomycin it is perhaps of some interest to note in connection with Dr. Estabrook's question that guanidine inhibits the arsenate-stimulated respiration of mitochondria.

LARDY: The experiments we have done with oligomycin parallel exactly those of Dr. Hollunger, and the compounds behave very much alike. However, there are discrepancies, e.g. endogenous ATP-ase is depressed by guanidine but DNP-stimulated ATP-ase is not completely depressed.

STRUCTURE AND FUNCTION OF
CHLOROPLASTS AND CHROMATOPHYTES

Chairman's Opening Remarks

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A glance at the list of distinguished speakers in today's proceedings quickly made me realize that it would be an act of supererogation if I attempted to discuss chloroplasts and chromatophores in general terms as an introduction to the session. I feel that it would be much more profitable if I made some general observations on one member of those inseparable photosynthetic twins—the carotenoids and the chlorophylls. Dr. Smith will discuss certain aspects of chlorophyll biochemistry, so I shall confine myself to the carotenoids. The invariable co-existence of carotenoids and chlorophylls in all photosynthetically active units strongly indicates an important function of carotenoids in photosynthesis. It has been known for a long time that they play an ancillary role in photosynthesis; they absorb light in the region of the spectrum least effectively used by chlorophyll and pass it on, with varying degrees of efficiency in different organisms, to chlorophyll for use in the primary photosynthetic act. This, however, does not make the carotenoids essential to photosynthesis, but only allows the more efficient use of the energy of the visible spectrum. However, the invariable association of carotenoids and chlorophylls in photosynthetic organisms suggests a more fundamental role than this. As Stanier [1] has put it, "In the long run natural selection ruthlessly eliminates non-functional gadgetry from living organisms and as biologists we may therefore be fully confident that the carotenoids of the photosynthetic apparatus are not merely the organic equivalent of tail fins". The work of Stanier and others strongly indicates that the essential function of carotenoids in the photosynthetic units is to prevent photo-oxidative damage by chlorophyll. I do not intend to discuss this further now, but no doubt various aspects of this work will be considered during today's session.

I wish to devote the remainder of my time to considering how carotenoids are synthesized. Carotenoids are one class of a wide group of natural products known as terpenoids; these have a common characteristic in that they are built up from isoprenoid (branched 5-C units). It is clear from the work of Lynen, Popják, and Bloch and their collaborators that the 5-C unit from which steroids and other triterpenes are formed is isopentenyl pyrophosphate (see Goodwin [2, 3] for details). The mechanisms involved in the formation of isopentenyl pyrophosphate from

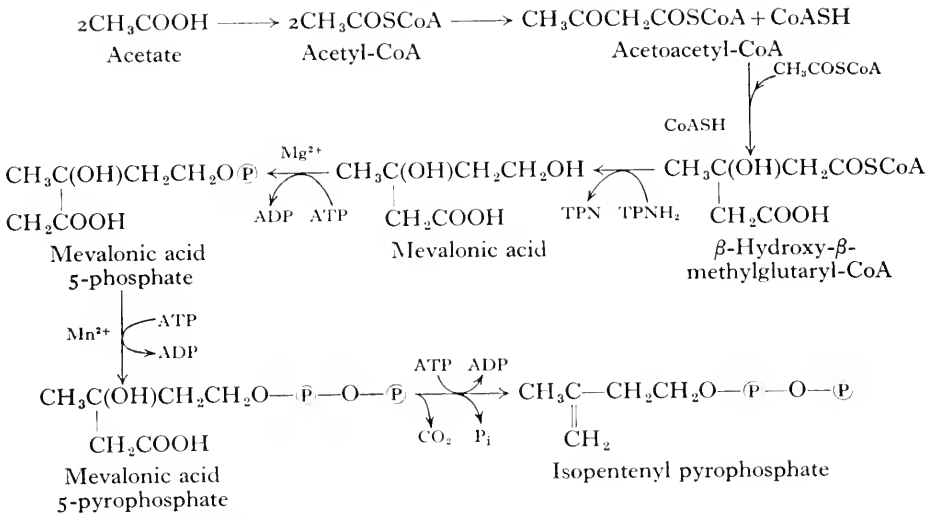


FIG. 1. The conversion of acetate into isopentenyl pyrophosphate.

acetate are outlined in Fig. 1. Experiments showing that isopentenyl pyrophosphate is also involved in carotenoid biosynthesis have not yet been reported; however, there seems little doubt that it is an intermediate because isotope experiments with various carotenogenic preparations have shown that acetate, β -hydroxy- β -methylglutarate and mevalonate are all incorporated in the expected manner (see Goodwin [2, 3].)

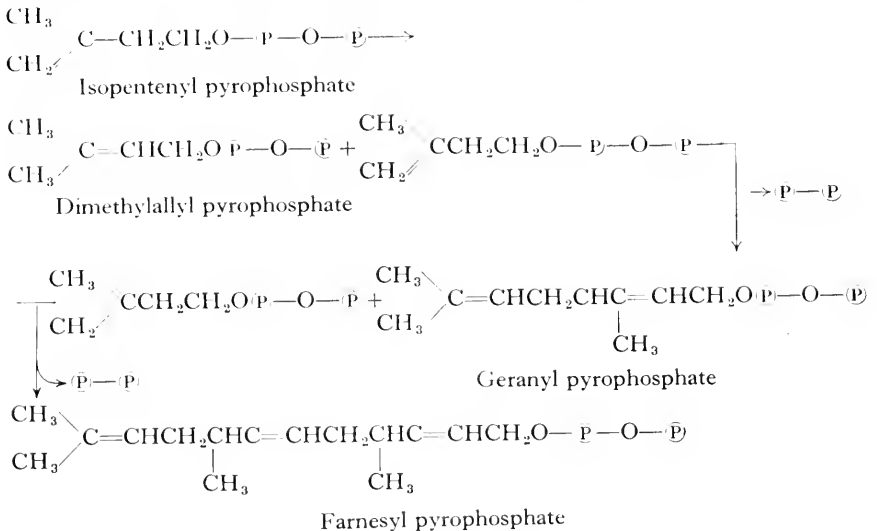


FIG. 2. The conversion of isopentenyl pyrophosphate into farnesyl pyrophosphate.

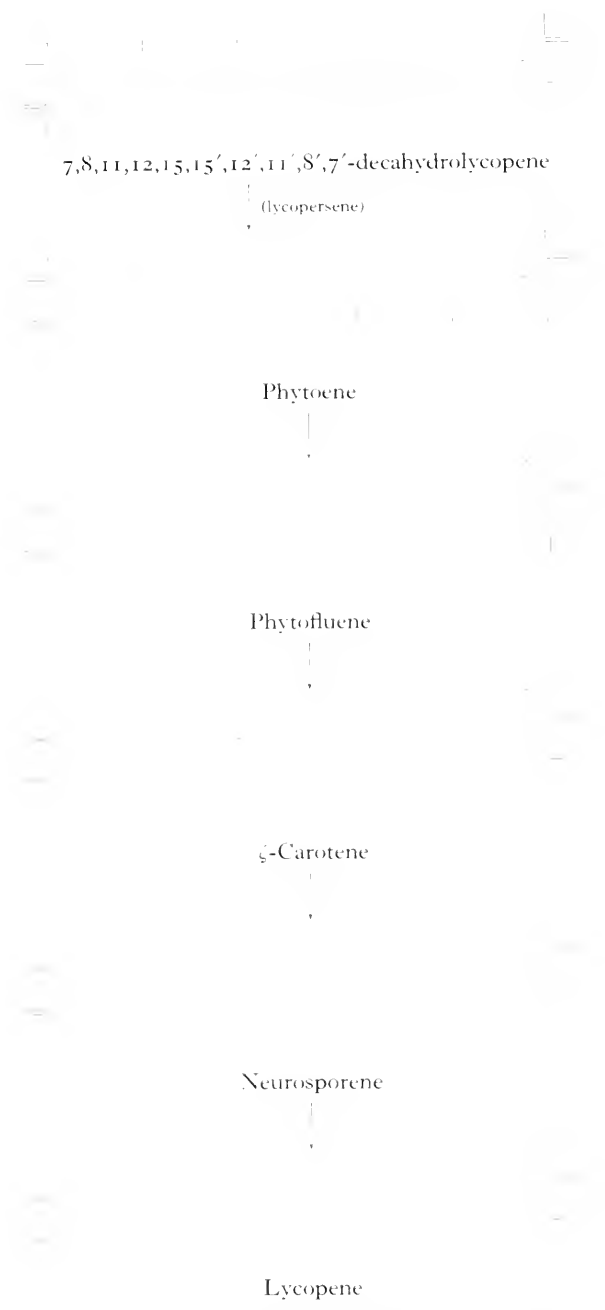
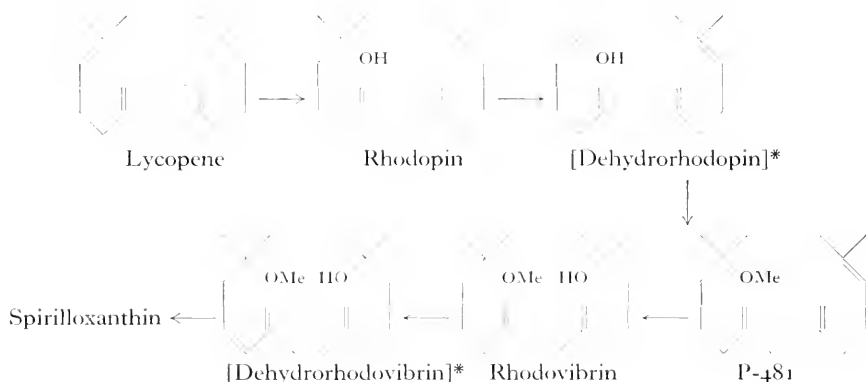


FIG. 3. The conversion of C-40 polyenes into lycopene.

In steroid biosynthesis isopentenyl pyrophosphate is first isomerized to $\beta\beta$ -dimethylallyl pyrophosphate which acts as a starter for polymerization. This compound then reacts with two molecules of isopentenyl pyrophosphate as indicated in Fig. 2, eventually yielding the 15-C compound farnesyl pyrophosphate which dimerizes to yield the hydrocarbon squalene, the acyclic steroid precursor. By analogy the corresponding compounds concerned with carotenoid biosynthesis would be the 20-C compound geranylgeranyl pyrophosphate and lycopersene respectively; neither has yet been unequivocally identified. If we accept the assumption that this lycopene derivative is the basic 40-C carotenoid precursor then three main problems arise which are unique to the biosynthesis of carotenoids: (a) what is the pathway of dehydrogenation of



* These compounds have not yet been identified.

FIG. 4. The conversion of lycopene into spirilloxanthin in *Rhodospirillum rubrum*.

the 40-C-precursor? (b) how do the acyclic (lycopene) and cyclic (β -carotene) derivatives arise? and (c) how do the oxygenated derivatives (xanthophylls) arise?

With regard to the first two queries lycopene almost certainly arises by the sequential dehydrogenation of phytoene via the route indicated in Fig. 3. The pathway of β -carotene synthesis is much less certain because of the doubts about the point at which cyclization takes place. My own view, which has been given in detail previously [4], is that cyclization is an early event in the conversion of the basic 40-C precursor into β -carotene.

Hydroxylated xanthophylls are formed in the photosynthetic bacteria by the insertion of oxygen following the completion of the parent hydrocarbon. For example, in *Rhodospirillum rubrum* the formation of spirilloxanthin from lycopene has been proved, and the pathway is probably that indicated in Fig. 4 [1, 3]. There are no reports of experiments indicat-

ing how hydroxylated xanthophylls are formed in higher plants and algae, but it is clear that β -carotene epoxides are formed in excised leaves by epoxidation of the parent hydrocarbon.

Having given a very brief summary of what we know about the mechanism of carotenoid formation, I shall conclude by mentioning two specific problems which should be of direct interest to this symposium. Both are concerned with the action of light on carotenoid formation in photosynthetic organisms and both are only in the embryonic state of development. The first problem is the synthesis of carotenoids in illuminated etiolated maize seedlings. Etiolated seedlings produce only small amounts of xanthophylls; on illumination they immediately begin to synthesize the typical plastid carotenoids, mainly β -carotene, lutein, and neoxanthin, along with the chlorophylls as the functional chloroplasts develop. Isotope experiments show that mevalonate and acetate are ineffectively incorporated into β -carotene during this period, but that CO_2 is specifically incorporated. Etiolated seedlings synthesize considerable amounts of sterols and mevalonate and acetate are incorporated into these compounds both in the dark and on illumination of the seedlings; CO_2 on the other hand is less effectively incorporated into the sterols than into the carotenoids [5]. We are now trying to find out the biochemical reason for the sudden switch of terpenoid precursors from steroid synthesis to carotenoid synthesis and for the effectiveness of CO_2 as a carotenoid precursor. A possible explanation is that TPNH_2 is required for the later stages of carotenoid synthesis (? dehydrogenation) and this would, of course, become available in increasing amounts in the developing chloroplasts. Furthermore, it has recently been observed (H. Yokoyama, personal communication) that in an enzyme preparation from *Phycomyces blakesleeanus* which incorporates labelled mevalonate into β -carotene and ergosterol, the addition of TPNH_2 to the suspending medium results in relatively more label appearing in the β -carotene fraction.

The second problem deals with the purple photosynthetic bacterium *Rhodospirillum rubrum*. When grown photosynthetically this normally produces in its unsaponifiable fraction a carotenoid spirilloxanthin and a terpenoid recognized at the moment only by its R_f value [6]. When *R. rubrum* is grown heterotrophically in the dark it is colourless and synthesizes only the terpenoid compound; on illumination spirilloxanthin and bacteriochlorophyll are synthesized together as functional chromatophores develop. This situation is obviously very similar to that encountered in etiolated seedlings. However, a somewhat different situation can be demonstrated under suitable conditions. Dr. June Lascelles in Oxford [7] showed that washed colourless *Rhodospseudomonas spheroides* resuspended in a medium containing small amounts of glycine, α -ketoglutarate, fumarate and salts, including Fe^{3+} , and with a gas phase containing 6% CO_2

oxygen and the remainder nitrogen, rapidly synthesized bacteriochlorophyll. Mr. Brian Davies in my laboratory has repeated these experiments using *Rhodospirillum rubrum* and found the same situation to exist; furthermore he has extended the experiments and has found what one would have anticipated, that spirilloxanthin is synthesized alongside bacteriochlorophyll. Two possible explanations exist: (a) either α -ketoglutarate and/or fumarate and the carbon residue of glycine are providing carotenoid precursors, or (b) the conditions are such as to stimulate the conversion of an existing precursor into spirilloxanthin. If the latter is the true explanation then the precursor must be far back in the biosynthetic pathway because resuspension of colourless *R. rubrum* cells in the Lascelles medium to which the carotenoid inhibitor diphenylamine has been added, results in the accumulation of the partly saturated polyenes, phytofluene, etc. If the first possibility is correct then compounds other than CO_2 are actively concerned in carotenogenesis and thus the situation is to some extent different from that in etiolated leaves. These differences of detail aside, the effect of light on both organisms is essentially the same, and it is hoped that soon a biochemical explanation of this important action of light in controlling terpenoid synthesis in photosynthetic organisms will be forthcoming. I think that you will agree that this is a key problem because, if this re-routing of terpenoid intermediates on illumination did not occur then functional chloroplasts and chromatophores would not result.

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Haem Protein Content and Function in Relation to Structure and Early Photochemical Processes in Bacterial Chromatophores*

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The essential process in photosynthesis is the absorption and conversion of radiant energy into chemical free energy with subsequent storage in a form which can be used for biosynthesis. Currently, the most widely studied example of this process in cell-free systems is "photophosphorylation"—the chromatophore, or chloroplast, catalysis of ATP synthesis from ADP and inorganic phosphorus, utilizing energy absorbed by the photoactive pigments contained in these particles.

The time during which the energy conversion and stabilization phase of photosynthesis occurs is subsumed between $\sim 10^{-9}$ sec. and $\sim 10^{-2}$ sec. The former limit is set by the time required for initial quantum absorption and subsequent migration of the energy packet to the active centre of the system by processes such as exciton migration, or induced resonance, which depend in detail on the specific structural features of the photoactive particles. It is possible that stabilization of the excitation energy in the so-called "triplet" state of the photoactive pigment can extend the upper boundary in time from 10^{-9} sec. to 10^{-4} sec. [1]. The latter limit is given by the average turnover number of enzymes involved in the biochemical processes which lead eventually from assimilation of CO_2 or organic material to synthesis of cell material.

We know very little about the photochemical processes which occur during this critical interval in time. A basic question which highlights this ignorance is: What molecular composition and placement is both necessary and sufficient to bridge the gap between quantum absorption and biosyntheses?

We may suppose that some sub-unit of the plant granum or bacterial

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chromatophore is a minimal structure. The usual experimental approach is to fragment cells to photoactive particles and then continue fragmentation until some photochemical process, such as the Hill reaction, or photophosphorylation, is no longer supported by the particle preparation obtained. Then, re-activation by the addition of external factors is used to define the biochemical system. Unfortunately, such an approach merely defines a system that can work, but not necessarily one that functions in normal photosynthesis. As an example, we may recall the remarkable activation of photophosphorylation by phenazine methosulphate, first seen in bacterial chromatophore preparations [2, 3, 4].

A refinement on this methodology is to examine soluble factors originally present in the intact cell, or chromatophore, and which were removed during fractionation. A number of investigators have found that the washings from chromatophores and chloroplasts contain activators for photophosphorylation, but the nature of these factors remains obscure. The soluble system obtained by washing fragmented chromatophores is complex, containing numerous enzymes associated with activities such as adenylate kinase, exchange of ATP with inorganic P, nucleotidase, nucleic acid depolymerase, catalase and peroxidase, etc. In addition, there are non-specific reductants, as well as flavins, quinones and haem proteins which have been dissociated from their binding sites in the chromatophore. Dr. Horio and I have found recently (unpublished) that thoroughly washed chromatophores from *Rhodospirillum rubrum*, which are wholly inactive in photophosphorylation, can be reactivated by addition of the purified haem protein, a pure yellow flavin enzyme which is a pyridine nucleotide-linked haem reductase, and a volatile reductant obtained by distillation of acetone extracts of fresh cells. These results are an improvement on those previously obtained using crude extracts, or artificial electron transport mediators like phenazine methosulphate, because they demonstrate the ability of single factors originally present in the chromatophore to participate in the normal metabolic process.*

Other approaches can be based on synthesis rather than breakdown of the photochemical apparatus. Possible methods include extraction of precursor particles from colourless mutants, physical treatments of normal cells which interfere with chloroplast or chromatophore development (e.g. heat [5], u.v. irradiation, variation in oxygen tension [6], heterotrophic growth conditions [6], etc.). Immunochemical approaches have been described in which sera specifically directed against components extracted from light-grown and dark-grown bacterial systems have been prepared [7, 8].

* H. Baltschetsky (*Biochim. biophys. Acta* **41**, 1 (1960)) has published recently results of studies of this type implicating flavin adenine dinucleotide as an intermediate in electron transport coupled to photophosphorylation.

However, despite the many researches, particularly on chloroplast development, emphasis has been mainly on morphology. Few chemical analyses at the molecular level have been performed. No structural studies have been conducted simultaneously with exhaustive molecular analyses. The development of the bacterial chromatophore system, the study of which is just beginning [9, 10], may provide a well-defined test system for future research.

There is one study available on molecular composition as a function of fragmentation. While the data are incomplete in many respects, they are sufficient, taken together with gleanings from various other researches in the literature, to base a valid discussion of possible relations between molecular composition and the primary processes in photosynthesis.

These data were obtained by Newton and Newton in our laboratory three years ago and are concerned with the composition of the photoactive sub-cellular particles derived by various fragmentation procedures from the obligate photo-anaerobe, *Chromatium* [11]. The gross composition and characterizations of some components of chromatophores and chromatophore fragments, as isolated by differential centrifugation, were studied. Qualitative kinetic analyses of the progressive fragmentation of cells into small subcellular aggregates were conducted, together with molecular analyses for each fraction.

I have recast these data so as to summarize briefly the essential results in a single table (Table I). These data may be expanded by borrowing some figures from other researches. Thus, Lester and Crane [12] give a figure of $2.9 \mu\text{M}$ Coenzyme "Q₇" (or "ubiquinone" [13]) per g. dry weight of cells. This approximates to $\sim 0.5 \mu\text{M}$ per g. wet weight of chromatophores, a relatively great quantity of this benzoquinone. For *R. rubrum*, a somewhat higher but comparable figure is given, the quinone found being "Q₉" and the concentration approximately twice that of the *Chromatium* "Q₇".

Inasmuch as all the photoactive structures known are supposed to be self-duplicating units, it can be expected that nucleic acids are present. As seen in Table I, acid-soluble nucleotides are found and in addition there is residual phosphate which is associated with protein and with insoluble nucleic acid. A reasonable treatment of these data indicates that out of the total P present ($85 \mu\text{M}$) probably no more than $20 \mu\text{M}$ can be ascribed to nucleic acid. This can be contrasted with the nucleic acid P content of chromatophores originally obtained from *R. rubrum* by Schachman, Pardee, and Stanier [14], who found for the same protein content a value of $\sim 200 \mu\text{M}$.

Of course it is not surprising that large variations in content of particular fractions will occur as the source of particles is varied. This is true of all fractions examined to date such as the chlorophylls [15], the caro-

TABLE I

MOLECULAR COMPOSITION OF *Chromatium* CHROMATOPHORES

(after Newton and Newton [11])

Based on 1 gm. wet weight of washed chromatophores

<i>Protein</i> (mg.)	166	(modified biuret reaction)
Cytochrome (μM)	0.18	(determined as pyridine hemochromogen)
<i>Carbohydrate</i> (mg.)	62	(anthrone reaction)
Acid-soluble	5	(mostly pentose)
Insoluble	57	(galactose polymer)
<i>Lipid</i> (mg.)	87	(mostly phospholipid; only base detected—ethanolamine)
<i>Pigments</i> (μM)		
Bacterio-chlorophyll	3.4	(spectrophotometric assay)
Bacterio-carotenoid	1.6	(spectrophotometric assay)
<i>Nucleotides</i> (μM)	9.5	(based on u.v. absorption as adenine)
Pyridine	0.2	(fluorimeter assay of TCA extract)
<i>Flavin</i> (μM)	0.17	
<i>Phosphorus</i> (μM)	85	
Acid-soluble	9.4	(8.5 μM of this fraction accounted for as inorganic P)
Insoluble	73	(51 μM of this fraction accounted for as lipid P)
<i>Iron</i> (μM)	12	(mostly non-haem)
Acid-soluble	5	(mostly present in ferrous form)

tenoids [16, 17], the haem proteins [18, 19] and the quinones [12]. The major finding appears to be that the bacterial chromatophores are relatively rich in RNA [14] and depleted in DNA [14], which suggests a basic composition like that of microsomes, as regards gross composition. In fact, the overall P distribution in various fractions of silver beet microsomes, as obtained by Martin and Morton [20], are much like those found by Newton and Newton [11] for *Chromatium* chromatophores. Results given by Nakamura, Chow, and Vennessland [21] for spinach chloroplast preparations also do not differ significantly from those reported for the chromatophores. The relation of nucleic acids to development of photo-active structures remains to be elucidated. A beginning has been made by Brawerman and Chargoff [5] whose interesting work I can only mention in passing because of time limitations. It is noteworthy, as far as photo-chemical function is concerned, that Nakamura *et al.* [21] reported extensive enzymic depolymerization of nucleic acid in chloroplast fragments failed to impair the photophosphorylation capacity. This indicates

that the relation between nucleic acid and the photochemical function is indirect.

The carbohydrate and lipid fractions deserve much more mention than I can give at this time. Briefly, the Newtons found (Table I) that in *Chromatium* chromatophores, the major fraction of the carbohydrate present was in the form of a polysaccharide, the monomer unit of which appeared to be galactose. The presence of a galactose moiety as a characteristic component of the photosynthetic carbohydrate fraction in both chloroplasts and chromatophores as well as a component found in galactosidyl lipids has been well-documented by Benson and his co-workers [21, 22]. Progressive fragmentation of the chromatophores to smaller fragments resulted in a loss of most of this polysaccharide with a corresponding relative increase in lipid [11].

The lipids present in most photosynthetic tissues appear to be predominantly of neutral or cationic type [22]. Mono- and digalactosyl monoglycerides predominate. There are also some new sulpho-lipids, one of which has been identified as a sulphonic acid analogue of the major plant glycosyl monoglyceride [23], e.g. the structure assigned by Benson *et al.* is 1-O-(1'-deoxy-1'-sulphoketopyranosyl)-3-O-oleoylglyceride. The basic phospholipid present in *Chromatium* appears to be almost wholly a cephalin—namely, ethanolamine phosphatidyl glycerol [11]. The nature of the fatty acids which are presumably bound as esters to the glycerol is still unknown. This phospholipid is held to account for practically all the fat in the *Chromatium* chromatophore [11, 24].

It seems evident that the photosynthetic structures elaborate special lipids and carbohydrates which in many cases appear unique to the photo-active particle systems. Very probably a major role involves stabilization of chromatophore and chloroplast structures which contain both polar and non-polar groupings. It may be mentioned that plastids from various plant sources appear to contain hydrolytic enzymes (phosphatidases) which attack lecithin and other lipids [25].

Major interest resides at present in another feature of data such as are exemplified in Table I. It will be noted that cytochrome (in this case, a cytochrome complex made up of a modified haem protein called "RHP" and a cytochrome of the *c*-type [26]) accounts for an appreciable fraction of the total protein. Thus, out of 166 mg. total, there are 0.18 μ M cytochrome. Most of this cytochrome is the "*c*" component which has molecular weight, as isolated in pure form, of 95 000 [24]. This means that approximately 17 mg., or 10% of the protein, is accounted for as cytochrome. In addition, there are trace amounts of haem proteins with which are associated catalase and peroxidase activities. A flavin component is associated to a major extent with a yellow enzyme which can be prepared from both *R. rubrum* [27] and *Chromatium* (R. G. Bartsch, unpublished)

and which, as mentioned previously, is a pyridine-nucleotide linked haem protein reductase.

These results relating to the cytochrome content of the *Chromatium* chromatophores are applicable generally to all photoactive particles, whether of bacterial or plant origin. Surveys of all the typical species of photosynthetic bacteria [28] and of a large variety of plants and algae [29, 30] reveal that, regardless of aerobic or anaerobic habit, these systems all contain relatively large amounts of haem proteins. Further, although the major component invariably is a cytochrome of the "c" type, no corresponding oxidase of the "a" type is found associated with chromatophores or chloroplasts. Significant aspects of these findings have been discussed sufficiently elsewhere [19, 31]. Let us proceed to the central topic of this paper—a possible relation between haem protein content and the early photochemistry of the photosynthetic process.

The ultimate consequence of the photochemical act may be thought of as the establishment of a voltage gap between two systems. This gap is sufficiently large in the case of the green plants and algae so that one system can operate at a "mid-point" potential reducing enough (negative E'_0) to drive reductive assimilation of CO_2 (and perhaps generate ATP simultaneously) while the other can provide a sufficiently high oxidizing "mid-point" (positive E'_0) potential eventually to liberate oxygen from water. In bacteria, a small gap may be all that is necessary because oxygen is not liberated during CO_2 assimilation. The significance of our question about a sufficient and necessary molecular composition and placement, posed in our previous discussion, is that if we know what molecules are present, their relative concentrations, and their disposition, we may begin to develop and examine hypotheses for identifying reactants in the primary photochemistry. In *Chromatium* chromatophores, Newton and Newton [11] have shown that the major constituents present in both chromatophores and chromatophore fragments include, in addition to the photoactive pigments and the major gross fractions of protein, lipid, and carbohydrate, components typical of a mitochondrial respiratory chain, e.g. pyridine nucleotides, flavins, quinones, and cytochromes. Associated with these compounds are a variety of enzyme activities typical of an electron-transport system, as noted previously.

In *Chromatium* chromatophores, there are, for every 20 bacteriochlorophyll molecules, 11 carotenoids, 1.5 haem protein, 1 flavin, and 1 pyridine nucleotide. We have remarked that further fragmentation to small particles results in the loss of a major part of the polysaccharides, some protein, but less lipid, so that the fragmented particles became relatively enriched in lipid. However, the haem protein content relative to chlorophyll remains unchanged, both doubling relative to total protein content. Thus, in the chromatophore fragment (which is still capable of

supporting photophosphorylation when incubated with certain external factors [3]), there are 40 bacteriochlorophylls, 17 carotenoids, 2.8 haem proteins, 0.5 flavins, and 1 pyridine nucleotide. Data on the quinone contents of the two preparations are not available. We may generalize these observations to the statement that similar molecules are present as major components in all photoactive structures.

Now, we may ask what mechanism we can assume for energy storage and which molecules of those mentioned as major constituents are likely reactants for production of molecular species sufficiently stable to couple to the biochemical phase of photosynthesis. Of course, there is little doubt that one reactant will be excited chlorophyll. The reactions it may undergo upon excitation are many but a most likely type of reaction is one involving electron transfer. It is not possible that electron ejection (photo-ionization) will occur because the quantum energy in actinic light is insufficient for such a process. However, electron donation, or acceptance, from a neighbouring molecule is possible. Some theories [32] are built on the notion that chlorophyll loses an electron to some acceptor and so becomes a strong oxidizing agent. An alternative notion is that it gains an electron and becomes a strong reducing agent. There is no way at present of deciding between these two alternatives.

On the basis of some arguments based on comparative biochemistry and the physical chemistry of the haem proteins (see later discussion in this paper) and results obtained by Duysens, Chance and others, using an approach based on differential spectrophotometry of fast reactions in suspensions of cells and extracts [33, 34, 35, 36], I have suggested [37, 38] that the primary electron transfer act involves reduction of chlorophyll by the iron haem protein complex, resulting in a reduced chlorophyll-chlorophyll couple on the one hand ($E'_0 \sim -1.0$ V.) and an oxidized-reduced haem protein couple on the other (Fig. 1). The potential developed depends on whether the oxidation of the central iron atom proceeds to a formal valence state of three positive, or whether it goes to a higher effective valence (+4 or +5, as in catalytic processes catalyzed by haem protein). In the former case, E'_0 will vary from ~ 0 to $+0.3$ V. In the latter, it may rise as high as $+1.0$ V. There is insufficient energy in the infrared quanta (~ 1.3 V.) effective in bacterial photosynthesis to provide the gap created by the reduced chlorophyll and oxidized Fe^{1+} or Fe^{5+} systems, which are separated by ~ 1.7 to 2.0 V., depending on what potentials are assumed for the reduced chlorophyll. Hence, it is not expected that the haem protein in the purple photosynthetic bacteria will be oxidized to a valence state higher than $3+$, so that the high positive potential required to liberate oxygen ($+0.8$ V.) is not reached. In this way, we may account for the absence of oxygen as a product in bacterial photosynthesis and for the requirement of an added H-donor, other than water. On this view

there is a cyclic process involving first photo-oxidation of haem, then thermal reduction by reducing equivalents supplied from the H-donor through a chain of intermediates.

The bases for these suggestions may be reviewed briefly. We know from a voluminous literature on electron transfer processes in systems containing organic metal conjugates or chelates, that the presence of a macrocyclic resonating system can induce rapid electron exchange between ions otherwise shielded by solvent [39, 40]. In all photoactive systems a situation exists in which an efficient resonating macrocyclic system—porphyrin or a derivative reduced porphyrin ring—is chelated to magnesium or iron as the central metal ion. If we suppose that the magnesium chelate (chlorin) is close to the iron chelate (haem), then excitation of the

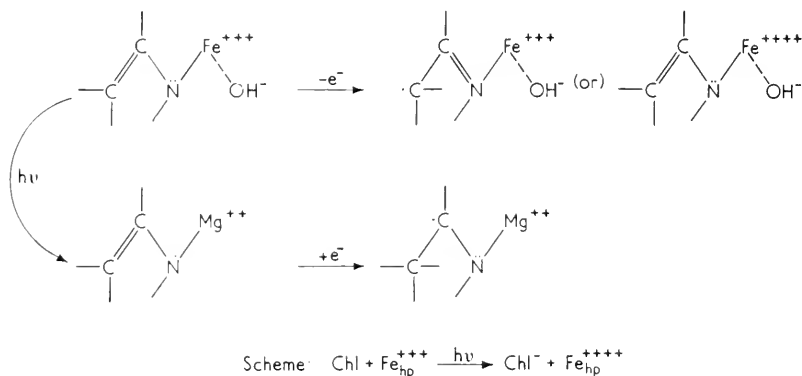


FIG. 1. Electron transfer reaction proposed as part of the primary photochemical process in photosynthesis.

magnesium chelate by a photon which gives rise to the characteristic red absorption band will result in an excited chlorophyll system with energy equivalent ~ 1.8 to 2.0 e.V. above the ground state. De-excitation can occur immediately by electron transfer from the neighbouring haem system. If the iron complex is one which is originally in the formal valence state of Fe^{n+} , it will be oxidized to a formal valence of Fe^{n+1} . Likewise, the chlorophyll acquires an excess negative charge which makes it equivalent to a "semichlorinogen" (see Fig. 1).

This process, which most probably leaves both products in their ground states, results in two systems separated in energy content by an amount close to the original energy of excitation of chlorophyll, the "mid-point" potential of the semichlorinogen system is more reducing than that of the oxidized haem system by ~ 1.8 e.V. Stabilization against back reaction may require $0.2-0.3$ e.V., so that we may assume safely a maximum of ~ 1.5 e.V. available for the spread in potential.

We can infer consequences of such a process by analogy with many observations available in the literature, even though nothing is known directly about solution chemistry of higher oxidation states of iron. George and Irvine [41] have shown that metmyoglobin treated by a variety of oxidizing agents (peroxide, permanganate, chloriridate) gives a product spectroscopically identical with the intermediate "complex II" formed when metmyoglobin acts as a peroxidase. They have established the E'_0 as $\sim +0.9$ V. This value is ~ 0.1 V. more oxidizing than that for the standard oxygen electrode. Hence, the oxidizing equivalents present in this complex can extract an electron from water. If it is assumed that the haem chelate-protein complex acquires a similar E'_0 , then the E'_0 for the semichlorinogen formed would be ~ -0.6 to -0.7 e.V., assuming 1.5 e.V. as the value of $\Delta E'_0$ between the reducing and oxidizing components. Such a strong reducing potential would be more than sufficient to provide an electron transfer step to pyridine nucleotide ($E'_0 = -0.3$) which could be coupled to formation of ATP from ADP and inorganic phosphate ("photophosphorylation") [42, 43]. On this basis, the "photoreductase" of San Pietro and Lang [44] would have assigned as its substrate the semichlorinogen as the photoreductant generated by the light reaction.

The reactions initiated by the presence of the Fe^{IV} -haem complex depend on the environment presented. A simple combination of chlorophyll and haem protein would have only the possibility of back reaction, or reversal of the process shown in Fig. 1. However, if an enzymic pathway (such as through the photoreductase to pyridine nucleotide) is available to remove the electrons from the semichlorinogen, then it can be expected there will be a preferential flow of electrons to the enzyme substrate. If a source of electrons is present in the haem complex, either in the protein or as a simple ligand (water), then reduction of the Fe^{IV} -haem to its original state would occur with the production of a free radical.

The evidence available from paramagnetic spin resonance studies of the metmyoglobin oxidation complex, while somewhat ambiguous, appears to be consistent with this postulated sequence of events. Gibson, Ingram, and Nichols [45] have shown that the complex, studied by George and Irvine and produced by peroxidation of metmyoglobin, exhibits an ESR signal with a g -value close to that for the free electron. The precise value for g is somewhat smaller than expected for a π -electron localized at a methine bridge carbon. It is more consistent with the presence of a delocalized electron in an orbital spread over the whole macrocyclic structure, or of a substrate free radical, such as OH. At the same time the signal at $g = 6$ corresponding to the unpaired electrons at the Fe site is quenched, indicating a change in the bonding at the metal ion site. George and Irvine [41] have presented evidence for this change as a production

of "ferryl iron" (FeO^{++}) or, alternatively, as in the formulation of Fig. 1. At present it is not necessary to postulate production of a ferryl complex which requires movement of two protons off the ligand water. This is indicated in Fig. 1 by leaving the iron in a formal valence state equivalent to Fe^{4+} , without alteration of the chemical nature of the ligand.

An alternative reaction scheme, which has been discussed by Calvin [46], begins with loss of an electron from excited chlorophyll, concomitant with generation of a positive hole in the chlorophyll complex. This postulate necessitates a delayed oxidation of the cytochrome, or at least reduction of some acceptor, such as pyridine nucleotide before oxidation of haem iron occurs. There is no conclusive evidence at present to refute this notion, although the low-temperature measurements of Chance and Nishimura [35] on the photo-induced oxidation of the *Chromatium* cytochrome system, together with the quantum yield data of Olson and Chance [36], seem to favour prior oxidation of haem iron as a primary reaction following quantum absorption.

A variety of interesting problems comes to mind when predictions are attempted for the chemical behaviour of a higher oxidation state such as postulated in Fig. 1. Fe^{4+} , which is isoelectric with Mn^{3+} would contain four unpaired electrons distributed in the five 3d orbitals of the metal ion. Upon combinations with the ligand groups, at least two could pair leaving two unpaired electrons and the two free 3d orbitals, so that the Fe^{4+} orbitals could hybridize as usual to give the octahedral complexes found for Fe^{3+} and Fe^{2+} . There is evidence from the studies on magnetic susceptibility of metmyoglobin-peroxide complexes that this occurs [47, 48]. If all the electrons paired, then seven orbitals rather than six would be available with the Fe^{4+} in a diamagnetic state. A ligand such as OH^- , but not H_2O , would favour such an arrangement, if analogy with the lower valence forms holds.

The stabilizing effect of both the porphyrin ring, and possibly the protein moiety, in a higher valence form can be inferred from many well-known examples such as the metal porphyrin complexes of silver, bismuth, cobalt, etc. Winfield and King have emphasized this possibility [49]. Dwyer [40] has discussed similar situations, especially the case of the nitroprusside ion, and it is from his discussion that the suggestion of a possible diamagnetic complex structure is drawn.

One point which should be made is that until direct data can be obtained on the chemistry of iron haem chelates when in a state of oxidation formally higher than Fe^{3+} , it is unsafe to assume that a molecule such as CO is specific for the Fe^{++} state. The criterion of a light sensitive CO-binding has been used universally to establish the presence of ferrous iron, but the possibility that Fe^{4+} could bind CO in a similar fashion is not excluded.

In terms of the chromatophore structure, we may visualize an aggregate of bacteriochlorophyll molecules [15] together with the accessory pigments such as carotenoids, which for the most part are not attached to molecules with which they can undergo irreversible electron transfer reactions upon excitation. Most of these chlorophylls upon excitation merely transfer energy by some obligatory mechanism, such as inductive resonance. Migration of the energy quantum proceeds through the pigment aggregate until a particular chlorophyll molecule is reached which can be de-excited by electron transfer in such a way as to produce the two electrochemical systems postulated above.*

Of all the molecules mentioned as analogues of the respiratory chain previously, the most plausible reactants which can produce both highly positive electrochemical systems while affording the possibility of stabilization are the haem proteins. They possess the necessary electron source—the metal atom—the necessary protein component for close coupling to the chlorophyll and the porphyrin ring for stabilization. Hence, we may assume it is the haem protein that reacts with the excited chlorophyll, rather than a quinone, a flavin, or a pyridine nucleotide.

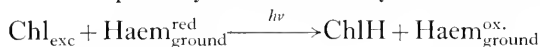
The rest of the reaction sequence requires that back reaction between the reduced chlorophyll and the oxidized haem be slow relative to the reduction by reduced chlorophyll of pyridine nucleotide or some other H-acceptor. This, as mentioned above, may be the role of the “photo-nucleotide reductase” discovered by San Pietro in chloroplasts. A similar enzyme may exist in bacterial chromatophores, but so far has not been found. It may be that the H-acceptor in the bacteria is not a pyridine nucleotide, but rather a SH-compound. The presence of large quantities of the yellow flavin enzyme which can not only function as a haem protein reductase, but also can show very great diaphorase activity [27] suggests that some SH-compound may be involved; on the basis of the recent demonstrations by Massey [50] and by Sanadi and Searls [51] regarding the possible coupling of SH-groups to flavin in diaphorase, it seems

* A possibility is that such a reactive site is chlorophyll dimer. S. S. Brody (see *Science* **128**, 835 (1958), also Brody, S. S. and Brody, M., *Arch. Biochem. Biophys.* **82**, 161 (1959)) have shown that in many plant systems an appreciable fraction of the chlorophyll is in the form of a non-fluorescent dimer. While it is not clear how such a complex could react to give two systems sufficiently stable and separated by a sufficient equivalent voltage, participation of such dimers in photochemistry certainly is not excluded.

It is also of interest that on the basis of an approach based wholly on analysis of fluorescence depolarization, G. Weber (see ref. [37], p. 408) has arrived at a scheme for the energy conversion mechanism in photosynthesis which is similar to the one proposed in this report in requiring resonance transfer to bring an excited electron in a chlorophyll singlet in contact with an electron donor. In later steps, he postulates separation and transfer of an electron from the chlorophyll-donor complex to an electron acceptor.

quite reasonable to suggest participation of an SH-compound or grouping.

At any rate, back reduction of haem by reduced chlorophyll would be slowed because both reactants would be expected to have reached their ground states after the primary deactivation by electron transfer, e.g.



Hence, some activation energy would be required to initiate the back reaction, despite the great energy difference of some 2 e.V. tending to drive it. The presence of a specific enzyme which would give the reduced chlorophyll the alternative of a reduction process requiring little or no excitation energy compared with the uncatalyzed back reduction of the oxidized haem could represent one of the stereochemical requirements for stabilizing the reduced product in the presence of the oxidizing system created by the electron transfer.

With the electron now located in some molecule at the reducing end of a "respiratory" chain, electron migration through the flavins, quinones, and various haem enzymes to the terminal oxidant, created by the initial photochemical electron transfer, would complete the cycle. As we will hear in the other papers, this type of electron transport coupled to the quantum excitation process is generally assumed to be the basic mechanism for photophosphorylation. An impressive, if not conclusive, accumulation of data is at hand to support this notion. Some of these data undoubtedly will be presented at this session.

An alternative scheme presented by Hill and Bendall [52] suggests that the phosphorylation step is coupled to a flow of electrons against the potential gradient between "tie points" on the respiratory chain represented by the haem proteins, in this case, cytochrome *b₆* and cytochrome *f*. The cytochromes are assumed to be involved in back reactions which restore the system to its original state before photo-excitation. Hill and Bendall consider this type of mechanism necessary because of the fact demonstrated by Arnon *et al.* [53] that photophosphorylation increases, rather than decreases, the yield of molecular oxygen in the chloroplast reaction. However, there are alternative explanations for this phenomenon, which do not require the concept of "reductive" phosphorylation. Thus, if phosphorylation occurs, as we have discussed, below the nucleotide level, rather than between nucleotide and haem, then the consumption of oxygen precursor assumed by Hill and Bendall as obligatory for photophosphorylation, will not occur. Rather the assumption required is that coupling of phosphate esterification to reduction of pyridine nucleotide increases the amount of pyridine nucleotide reduced and hence of oxygen precursor formed.

There are a few points I think need brief discussion relating to the generalization of haem proteins as H-donors in the fundamental photo-

chemical process. There are abundant data showing that in *Chromatium* [36] and in *R. rubrum* [54] the primary oxidation involves the cytochrome *c*-type haem protein which has been isolated, purified, and characterized in our laboratory [26, 27]. The evidence includes not only kinetic studies in the presence and absence of a variety of inhibitors, but also the demonstration that the cytochrome oxidation involves several components, one of which is oxidized as rapidly at -180° as at room temperature [35]. Data for comparable changes in oxidation state of chloroplast haem proteins remain meagre [55].

Now, it may be that there are qualitative differences between plant and bacterial photosyntheses, primarily owing to the ability of the former to produce molecular oxygen. There certainly may be factors not considered in the previous discussion, which are of crucial importance in the process of oxygen production. One possibility is the metal, manganese, which is present in very large amounts in chloroplasts, and which appears to be required for green plant photosynthesis, whereas it does not seem essential (at least in more than trace amounts) in bacterial photosynthesis [56]. Kessler has presented some preliminary evidence [57] correlating manganese with the oxygen-producing system. Very recently, Treharne, Brown, Eyster, and Tanner [58] have found that an electron spin resonance arising from manganese ion in *Chlorella* kept in the dark disappears upon illumination, and that this phenomenon can be linked with a photo-oxidation of Mn^{++} .*

It is also known from a discovery by the late R. Emerson that two quanta can co-operate over relatively long time intervals to increase the yield of molecular oxygen. In the chromatophore there is a relatively small ratio of chlorophyll to protein. From Table I we can see the ratio of chlorophyll to cytochrome is ~ 15 . This ratio is usually greater than several hundred in most chloroplasts [15]. Similarly the ratio of chlorophyll to pyridine nucleotide is 20 in the *Chromatium* chromatophore, whereas it can be no less than 2500 in spinach chloroplasts [59]. This greatly increased ratio of chlorophyll to other components in oxygen

* We may recall, if only in a footnote, the remarkable reaction, first noted by R. H. Kenten and P. J. G. Mann in 1949 and studied since by them (see *Biochem. J.* **45**, 255; **46**, 67; **52**, 125; **61**, 279) in which manganous ion is oxidized photochemically in the presence of plant peroxidase, hydroperoxide, and a peroxidase substrate, such as a monohydric phenol. Pyrophosphate is added to trap the manganic ion formed as the insoluble manganic pyrophosphate. These authors have found that chloroplasts can catalyze this reaction, and suggest that in photosynthesis a cycle occurs involving alternate photo-oxidation of manganous ion to manganic and reduction by plant material of manganic to manganous. W. F. Andreae (see *Arch. Biochem. Biophys.* **55**, 584) has determined that this reaction, which depends on the presence of catalase or peroxidase, can be induced by catalytic amounts of a hydrogen donor in the presence of a variety of light sensitizers. He has noted further the nature of hydrogen donors most effective in catalysis.

producing systems may be a consequence of the need to funnel more than one exciton to a given reaction site to produce molecular oxygen. It is reasonable to suppose that in a process involving multiple electron donation, as in the production of molecular oxygen, a mechanism for delivering the energy of more than one quantum to an active site may be required.

Perhaps the puzzling inability of the green sulphur bacteria to produce molecular oxygen, despite their utilization of quanta with energies as high as those absorbed effectively by green plants, is owing to relatively low chlorophyll content.

Returning to haem protein function in photosynthesis, the failure to observe shifts in spectra in the chloroplast upon illumination which can be interpreted as oxidation of haem, can be rationalized on the basis of the reaction scheme of Fig. 1. The spectroscopic methods employed at present permit only observation of changes associated with the ferrous to ferric transition. Transitions from ferric haem to ferryl or pentavalent iron haem do not involve changes in characteristic maxima in difference spectra which are sufficient to allow detection by present procedures. If the cytochrome *f* (chloroplast cytochrome *c*) is in its ferric state to begin with, then the photo-oxidation may proceed to the higher valence state of iron, required for generation of the system which oxidizes water, without being accompanied by a visible shift in absorption.

Leaving sheer speculation for the more solid ground of physical chemistry, it should be emphasized that our knowledge of the chemical potentialities of haem proteins is limited; it is derived solely from studies of specimens obtained from a restricted set of unique biochemical structures—the mitochondrial respiratory systems. As discussed elsewhere [60] haem proteins derived from a variety of bacterial and plant sources, where metabolism is in no way associated with obligatory reduction of oxygen, exhibit a great diversity of physico-chemical properties quite unexpected on the basis of the classical cytochrome preparations. There is a great urgency to isolate in pure form in sufficient quantities as many of these haem proteins as possible to enable intensive chemical studies.

As an example, I may cite the unusual haem protein we know as "RHP", which appears to be present only in the purple photosynthetic bacteria [19]. R. J. P. Williams has presented some elegant studies on haem models from which he has been able to make some remarkable correlations between oxidizing potential, spectra, magnetic properties, and haem binding and structure in the haem proteins [61]. RHP represents a class of haem protein, hitherto unknown, which can be rationalized in the Williams scheme, provided one of the ligands in the co-ordination position out of the porphyrin plane is a group with a relatively high proton affinity (e.g. carboxyl, hydroxyl, etc.). RHP is a myohaematin protein with a typical myoglobin-like spectrum and electro-

chemical potential. It cannot bind oxygen reversibly, but appears to function as an oxidase or as an electron carrier in the photorespiratory chain [27]. It contains 2 haems per molecule (MW = 28 000–35 000, depending on the source). One or both of these haems may be bound by only a single thio-ether linkage such as is characteristic of cytochrome *c*, which has two such links to a single haem.

It is evident that this protein provides a good test object for the present theories about haem protein structure. For instance, Williams has predicted RHP would be a "high-spin" complex [62]. His prediction seems to be correct on the basis of work by A. Ehrenberg (unpublished) using a crystalline sample of pure RHP provided by Dr. Horio and myself.

Further work on the amino acid sequence of haem peptides obtained from RHP as well as from other bacterial cytochromes, should provide important data for rationalizing the structural aspects of haem protein chemistry, and is now proceeding in our laboratory. We expect that work on the bacterial and plant cytochromes will greatly modify and extend present concepts of the chemistry inherent in the combination of iron tetrapyrrolic chelates and proteins.

However, it is unlikely that any future developments will support a notion, such as put forward by Arnon [63], that chloroplast, or any, cytochrome in its Fe^{3+} state, will possess sufficient positive electrochemical potential to extract electrons from the hydroxyl ion or water. Hill and Bendall [52] point the fallacy of this notion properly in reference to the cytochromes known at present to exist in chloroplasts—namely, the *c*-type haem protein, cytochrome *f*, and the *b*-type haem protein—cytochrome *b₆*. George and Irvine have found [64] that mammalian cytochrome *c*, as a representative of the haemochrome type haem proteins, does not react with strong oxidizing agents to give the higher valence (Fe^{4+}) form of haem, as appears to be the case with peroxidase or metmyoglobin. It would seem, then, that we must search for the sort of haem protein postulated in the scheme of Fig. 1 among the haem compounds of plant chloroplasts which are contained in the haem fraction which is not accounted for as either cytochrome *f* or cytochrome *b₆*.

So far, the only plausible haem compound found which resembles myoglobin and other myohaematin proteins is the RHP of the bacterial chromatophores. Its presence in chloroplasts remains to be demonstrated. However, even if a myoglobin-type compound is absent, there are still both peroxidases and catalases present in appreciable quantities in chloroplast tissues; any of these may reveal the requisite properties upon isolation and purification.* Even the attainment of a Fe^{4+} state in cytochrome *f* is

* It is possible that the peroxidase and catalase activities found in chloroplasts are functional, at least in part, in the manganese cycle suggested by Kerten and Mann (see previous footnote).

not excluded. In the reactions studied by George and Irvine movement of two protons is required and, most likely, a "ferryl" (FeO^{++}) state is formed. This is not required in a photo-induced electron transfer reaction of the type shown in Fig. 1. It may be, therefore, that the only way to reach the Fe^{4+} state in the chloroplast cytochrome, or other haem protein, is by a photochemical oxidation which proceeds by electron transfer unaccompanied by proton transfer.

The search for new haem proteins in photosynthetic tissues and intensive study of their structures should be intensified. At the same time, experiments designed to reveal photochemical capacities of haem proteins should be pursued. The present status of knowledge about haem proteins seems well suited to application of Charles Darwin's admonition that "without speculation there is no good and original observation".

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Discussion

CHANCE: This is a very exciting mechanism which Dr. Kamen has presented and I am sure that it is just as he says: without speculation we can't get anywhere. But I want to pull him back a little bit if I may because he has galloped off with the wrong haem protein. I think this mechanism is intuitively based on RHP being the initial electron donor and I think the initial electron donor is cytochrome *c*. In studies of *Chromatium* even at temperatures of liquid nitrogen we observed on illumination of the bacteria the disappearance of a band which has a peak at 420 m μ which suggests that the primary event involves a *c*-type cytochrome and not RHP.

KAMEN: What I was actually talking about was oxygen evolution. In the bacterial chromatophores, we do not have oxygen evolution so there is no necessity to go to the higher valency states. You just start with the Fe^{2+} and go to the Fe^{3+} . I did say that the system produced in bacteria was not sufficiently electropositive to produce the Fe^{4+} state. One could suppose that if in green plants cytochrome begins in the Fe^{3+} state, you won't see the change because it involves going from Fe^{3+} to Fe^4 , whereas in the bacteria which are anaerobic, the steady state of the cytochromes is predominantly Fe^{2+} . The cytochromes would be reduced in the dark, and when you add light you would go from the Fe^{2+} to the higher valency state, maybe even Fe^{4+} for all I know, but certainly to Fe^{3+} and you would see the usual difference spectrum. I should say that Dr. Chance's group has provided the best evidence for this reaction in the bacteria by showing that it goes at liquid air temperature. As regards the RHP question I should say that cytochrome *c*-type proteins cannot be peroxidized to Fe^4 . Philip George tried this with cytochrome *c*: you take cytochrome *c* and add permanganate to it and all you get is destruction of protein, unlike what happens with myoglobin, so I think this makes it improbable that cytochrome *c* is involved in the primary reaction. I think it must be something like RHP, or even something quite remote from a haem protein like manganese. I don't think there is any way of telling at this time. I think we should be looking for a myohaematin-type protein in green plants. There are catalases and peroxidases present in chloroplasts and chromatophores. These may be the things which are reacting in trace amounts, and, if they are you might never see a spectrum corresponding to the reactions of these components but rather only the results arising eventually from oxidation of cytochrome *c*.

Observations on the Formation of the Photosynthetic Apparatus in *Rhodospirillum rubrum* and Some Comments on Light-Induced Chromatophore Reactions*

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Formation of photochemically active chromatophores in the dark

Cells of *Rhodospirillum rubrum* when cultured aerobically in the dark were found to be free of chromatophores by Schachman *et al.* [1]. Vatter and Wolfe [2] and Hickman and Frenkel [3] confirmed this observation. More recently, Cohen-Bazire *et al.* [4] demonstrated that when this organism was grown at very low oxygen tensions it was capable of producing chlorophyll. In a more detailed study Cohen-Bazire and Kunisawa [5] studied chlorophyll formation in dark- and light-grown organisms and also measured light-induced phosphorylation carried out by chromatophores isolated from these organisms. In the study presented here we have concentrated on the effects of oxygen tension on growth, chlorophyll formation, and on structure of dark-grown organisms. We present some preliminary observations [6] which we intend to expand and present in greater detail at a later time.

EFFECT OF OXYGEN TENSION ON GROWTH AND CHLOROPHYLL FORMATION BY DARK-GROWN CELLS

In a previous study [7] it was observed that *Rhodospirillum* grown either in the dark or in the light had a pronounced CO₂ requirement which, except for large additions of either yeast extract or casein hydrolysate, could not be replaced by many common metabolic intermediates which were added either singly or in various combinations to CO₂ free culture media. This CO₂ requirement was found to saturate at about 1% CO₂, and all gas mixtures employed subsequently were enriched with either 1 or 5% CO₂. In our first experiments dark-grown cultures were gassed

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with air enriched with CO_2 , and the rate of chlorophyll formation and growth was varied by adjusting the flow of air through the spargers of the culture tubes. With this method, however, it was difficult to obtain reproducible results, except for the conditions when the cultures were gassed rapidly* with air or with nitrogen. To obtain more reproducible results we secured the following gas mixtures: 0.5, 1.55, and 7.3% oxygen in nitrogen supplemented with 1% CO_2 (the values for oxygen indicated here were obtained by mass spectrometric analysis). All cultures were gassed at sufficiently rapid rates* so that a further increase in gassing

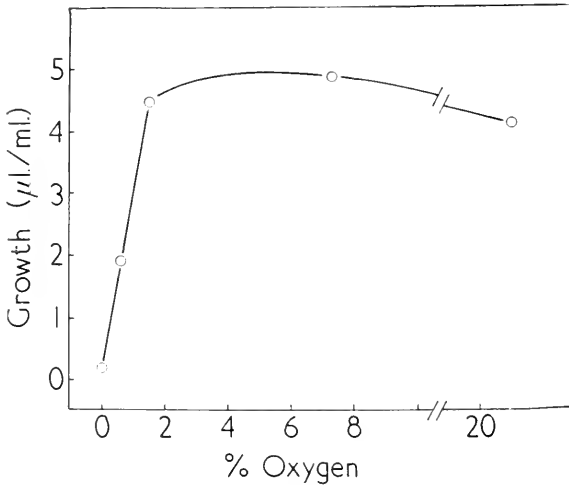


FIG. 1. Effect of oxygen tension in the gassing mixtures on growth of *R. rubrum* in the dark (based on change in packed cell volume per ml. of culture suspension) measured four days after inoculation. Initial concentration 0.11 $\mu\text{l.}$ of packed cells per ml. of culture suspension. Rate of gassing through spargers at 35 ml. of gas mixture per minute per 40 ml. of culture suspension in 100 ml. culture tubes. Culture tubes incubated at 30°.

rates caused little if any effect on rates of growth and chlorophyll production.

Figure 1 shows the effect of oxygen tension in the gassing mixtures on total growth per culture as measured after four days of incubation in the dark. Growth was measured by measuring changes in cell mass or by measuring changes in turbidity at 680 $m\mu$ calibrated against packed cell volumes of aliquots of the culture suspensions which were centrifuged for 35 minutes at 2000 times gravity in Hopkins vaccine tubes. While growth saturates at about 2% oxygen (Fig. 1), chlorophyll concentration on a cell volume basis decreases with increasing oxygen tensions and reaches

* 35-40 ml. of gas per minute per 40 cm^3 of liquid volume.

practically zero at 7-8% oxygen in the gassing mixture (Fig. 2). A combination of the curves in Figs. 1 and 2 results in the curve of Fig. 3 which shows that chlorophyll concentration per volume of culture reaches a

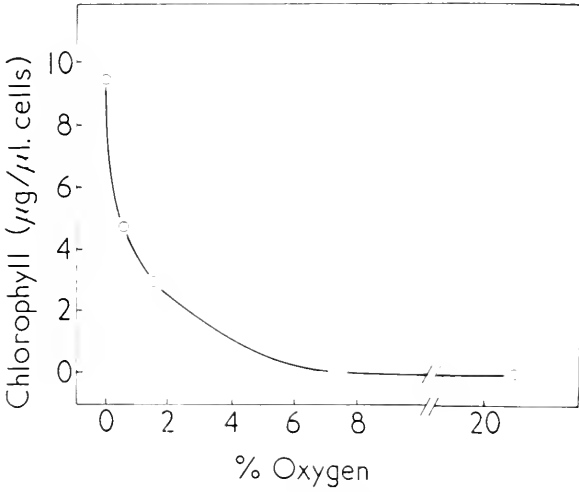


FIG. 2. Effect of oxygen tension in the gassing mixtures on the chlorophyll content (in μg bacteriochlorophyll per μl . of packed cells) of dark-grown cells measured four days after inoculation. Original inoculum contained $0.024 \mu\text{g}$. bacteriochlorophyll per μl . of packed cells. Culture conditions identical with those indicated for Fig. 1.

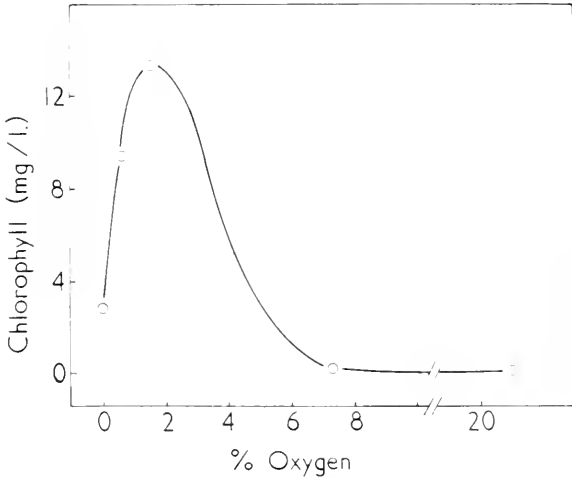


FIG. 3. Effect of oxygen tension in the gassing mixtures on the chlorophyll content per litre of bacterial culture suspension incubated in the dark, measured four days after inoculation. This graph represents a combination of Figures 1 and 2.

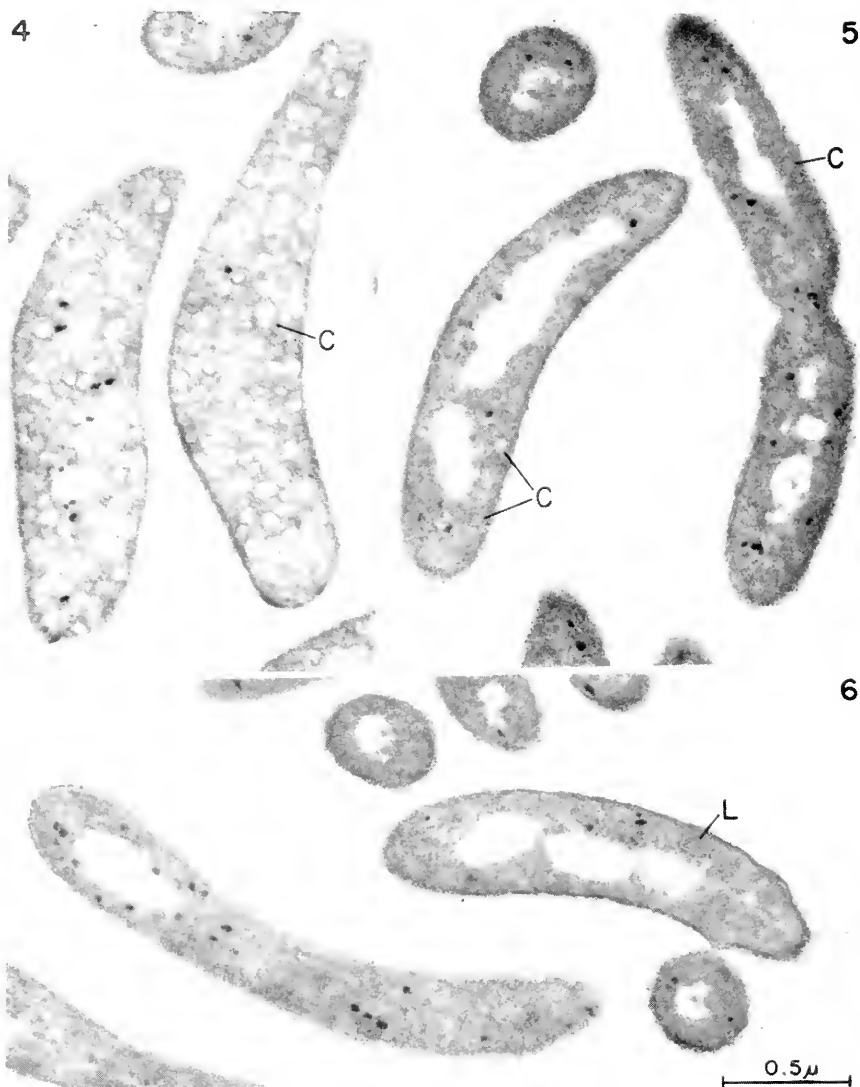


FIG. 4. Sections of cells cultured anaerobically in the dark for 96 hours at 30° , gassed with 5% CO_2 in N_2 (residual O_2 : 0.036%) at a rate of 35 to 40 ml. of gas per hour (cf. Fig. 14, ref. [3]). The bacteriochlorophyll content at the end of the period of 9.5 μg . per μl . of packed cells. The culture had been derived from an inoculum of almost colourless aerobically dark-grown cells which contained 0.024 μg . of bacteriochlorophyll per μl . of packed cells. The section of the anaerobically cultured cells shows abundant and distinct chromatophores (C). Magnification as indicated for Fig. 6.

FIGS. 5 and 6. Sections of cells cultured for 66 hr. aerobically in the dark at 30° (cf. Fig. 9, ref. [31]). The cultures were gassed with 5% CO_2 in air; the gassing rate was somewhat slower than indicated in the text and synthesis of a small amount of chlorophyll had taken place. A few scattered chromatophores (C) are apparent in some of the cells. A lamellar system (L) of unknown composition and function (cf. Figs. 21-25, ref. [3]) also can be observed.

maximum of about 2% oxygen even though chlorophyll concentration per unit volume of cells is highest under near anaerobic conditions (0.036% O₂) (Fig. 2). The observed maximum in chlorophyll concentration per volume of culture at about 2% is due to the rapid growth of the organism at this oxygen tension where chlorophyll synthesis is only partly inhibited. As the oxygen tension is raised beyond this level, there is no appreciable change in the amount of growth but chlorophyll synthesis is increasingly inhibited and comes to a standstill at about 8% oxygen.

STRUCTURE OF DARK-GROWN CELLS AS REVEALED BY ELECTRON MICROSCOPY

Electron micrographs of thin sections of cells grown in the dark at various oxygen tensions reveal the following picture. Under highly aerobic conditions the nearly complete absence of chlorophyll is accompanied by a virtually complete lack of chromatophores in the sectioned cells (Figs. 5, 6). Cells derived from near anaerobic cultures gassed with nitrogen containing 0.036% oxygen and 5% carbon dioxide contained a high concentration of bacteriochlorophyll (Fig. 2) and when sectioned revealed a great abundance of chromatophores (Fig. 4). It was possible to isolate photochemically active chromatophores from such cells (Table I) which could carry out both light-induced formation of ATP* and the photo-reduction of DPN in the presence of suitable cofactors. Observations on cultures grown at intermediate oxygen tensions indicate a decrease in chromatophore concentration with increasing oxygen tension. It appears that both chlorophyll concentration and the abundance of chromatophores can be controlled within certain limits by controlling the oxygen tension at which the organisms are grown in the dark.

PHOTOCHEMICAL ACTIVITIES OF DARK-GROWN CELLS AND OF CELL- FREE PREPARATIONS DERIVED FROM THEM

Intact cells, derived from initially chlorophyll-free cells, which had developed chlorophyll in the dark at reduced oxygen tensions, show an active CO₂ fixation (Table I). Cell-free preparations obtained from such cells carry out active light-induced phosphorylation and the photo-reduction of diphosphopyridine nucleotide [6, 8]. Cohen-Bazire and Kunisawa [5] who have carried out similar work with *R. rubrum* also have

* Abbreviations used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; P_i, inorganic orthophosphate; DPN, DPNH, diphosphopyridine nucleotide and its reduced forms; TPN, triphosphopyridine nucleotide; FMN, FMNH₂, flavin mononucleotide and its reduced form; PPNR, photosynthetic pyridine nucleotide reductase.

examined rates of light-induced phosphorylation and have reported rates for preparations from both dark- and light-grown cultures which are a good deal higher than the ones reported here, and in fact appear to be the highest ones reported in the literature for bacterial photo-phosphorylation.

Vernon and Ash [9] have reported that light-induced reduction of DPN by extracts from *R. rubrum* is increased in the presence of magnesium ions. We have also observed a small stimulatory effect of magnesium (or manganese) ions on the photoreduction of DPN. We have noticed, however, that this effect is much more pronounced with cell-free preparations

TABLE I

PHOTOCHEMICAL ACTIVITY OF INTACT CELLS OF *Rhodospirillum rubrum* AND OF CHROMATOPHORES DERIVED FROM CELLS CULTURED IN THE DARK AND LIGHT

	Initial rates (at light saturation):	
	$\frac{\mu\text{M}}{\text{hr.} \times \mu\text{M bacteriochlorophyll}}$	
	Preparations from cultures grown in the	
	Dark	Light
A. Intact cells:		
CO ₂ uptake	30*	—
B. Isolated chromatophores:		
(a) ATP formation	18.4	21.5
(b) DPN reduction:		
+ Mg ⁺⁺ (final concn.:		
1.3 × 10 ⁻³ M)	12.9	21.6
- Mg ⁺⁺	6.8	18.8

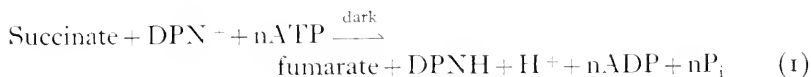
* Corrected for CO₂ production.

from dark-grown cells (Table I). Repeated washing of the dark-grown preparations almost completely eliminates the photoreducing activity, but such activity can be restored (more or less completely) by the addition of magnesium (or manganese) salts. Repeated washing of preparations obtained from light-grown cells produces a much more gradual loss of activity which can be restored by the addition of magnesium ions. We believe that this behaviour toward magnesium (or manganese) may reflect some subtle differences in the particles derived from light- and dark-grown cells, indicating that either less magnesium (or manganese) is bound by the chlorophyll containing particles from dark-grown cells, or that it is leached out more easily. We hope to obtain more information about this ion effect in the course of work on the development of the bacterial chromatophore.

Some comments on light-induced pyridine nucleotide reduction by bacterial chromatophores

REDUCTION OF PYRIDINE NUCLEOTIDES BY CHROMATOPHORES AND BY MITOCHONDRIA

In line with the discussion carried out by Dr. Chance and by other participants at this meeting on the reduction of pyridine nucleotides by mitochondria in the presence of ATP (reaction 1) (10-12),



one may compare this reaction with the light-induced reduction of diphosphopyridine nucleotide carried out by *R. rubrum* chromatophores which carry out the following reactions [8, 13-15]:



Consequently, it might be postulated that the photoreduction of DPN (reaction 4) by *R. rubrum* chromatophores could be due to the participation of ATP formed according to reaction 2, and that the ATP thus formed would be utilized for the dark reduction of DPN according to reaction 4. It is, therefore, of interest to examine the existing evidence for and against such a reaction scheme involving a combination of reactions 2 and 1 to account for the light-induced reduction of DPN by bacterial chromatophores.

The photoreduction of DPN (reaction 4) by bacterial chromatophores and the dark reduction of DPN (reaction 1) by mitochondria have the following characteristics in common: both require DPN, succinate and possibly Mg^{++} (or Mn^{++}), while ADP appears to be inhibitory to DPN reduction in both systems, although there is not complete agreement on the behaviour of the chromatophore system, as will be described later. The two reaction systems appear to differ in the following respects: (a) the mitochondrial system requires ATP, but such a requirement has not been established for the chromatophore system, although the participation of endogenous ATP cannot be excluded at the present time; (b) it appears that added ATP will not substitute for light in bringing about a dark reduction of DPN by washed *R. rubrum* chromatophores [15]; (c) in mitochondria there is a direct relationship between the amount of DPN

reduced and the amount of ATP utilized in the reaction. Chromatophores in the light may form only ATP (reaction 2), or only reduced DPN (reaction 4), or both ATP formation and DPN reduction may occur simultaneously in the presence of the required cofactor for both reactions. Vernon and Ash [13] have studied reactions 2 and 4 in some detail and have found that the amount of inorganic phosphate esterified in the light was the same regardless of whether their preparations carried out a simultaneous reduction of DPN, and they concluded that the light-induced phosphorylation reaction and the photoreduction of DPN occur independently of each other. We have observed, on the other hand, that the rate of photoreduction of DPN is inhibited under conditions where the preparations carry out the light-induced formation of ATP at the same time [15], indicating a possible relationship between these two processes, but this interaction appears to be different from the one exhibited by mitochondria. Only a more detailed analysis of the kinetics of the chromatophore reactions can clarify the conflicting reports in the literature.

As mentioned earlier there is disagreement about the effect of ADP on the photoreduction of DPN by *R. rubrum* chromatophores. Vernon and Ash [9] initially reported that ADP inhibited the photoreduction of DPN; in a later paper [13] such an inhibition was not observed. We have noticed, however, that ADP alone does not bring about an inhibition of DPN-photoreduction. A marked inhibition is observed only when inorganic phosphate and Mg^{++} are added and the preparation carries out active photophosphorylation [15].

The observations available thus far would indicate that the light-induced reduction of DPN by *R. rubrum* chromatophores may be achieved without the utilization of ATP. Except for the requirement of an exogenous reducing agent [16] and the absence of oxygen production, this reduction appears to be more closely akin to the photoreduction of pyridine nucleotides by chloroplasts than to the dark reduction carried out by mitochondria.

PHOTOREDUCTION OF PYRIDINE NUCLEOTIDES AND THEIR POSSIBLE ROLE IN METABOLIC REGULATION

On several occasions Dr. D. I. Arnon has raised the question as to the curious specificity of the purified photosynthetic pyridine nucleotide reductase (PPNR) of San Pietro for triphosphopyridine nucleotide [17]. The specificity of *R. rubrum* chromatophores for diphosphopyridine nucleotide is equally puzzling [15, 18]. It may, therefore, be of interest to consider whether these observations can be brought in line with recent views on the role of these two pyridine nucleotides in metabolic regulation which have been reviewed most recently by Klingenberg and Bücher [19].

The concept has developed in recent years that TPNH furnishes reducing power to a great many synthetic reactions in metabolic pathways (ref. [19], Table IV), while DPNH represents the prime energy source for oxidative phosphorylation carried out by mitochondria, and thus only indirectly supports and controls a great variety of synthetic metabolic reactions through the production of ATP.

In oxygen-producing plants there is an obvious relation between the TPN specificity of the PPNR and the TPN specific triosephosphate dehydrogenase present in leaves [20-23], as has been pointed out by Arnon [24]. Is it possible that the primacy of TPN reduction over DPN reduction by green plant photosynthesis makes it feasible to channel photosynthetic reducing power more effectively into many biosynthetic pathways in addition to those of carbohydrate synthesis? This primacy of TPN photoreduction over that of DPN, in oxygen-producing plants, may represent an important evolutionary advance over the situation that exists in *Rhodospirillum*, where, at least *in vitro*, isolated chromatophores specifically photoreduce DPN.

It remains to be seen whether there is any relevance to the hypothesis proposed. A beginning has been made in studies on the effect of light on oxidized and reduced pyridine nucleotides in green plants [25], and on the metabolic fate of hydrogen in illuminated algae [26], but comparisons with photosynthetic bacteria are not yet available. One thing we do know is that there appears to be a much closer relation between respiration and photosynthesis in the non-sulphur purple bacteria than there is in most oxygen-producing plants [27-30]. While the reasons for this can be manifold, the pyridine nucleotide specificity in light-induced reactions may represent an important aspect in considerations of over-all metabolic control in photosynthetic organisms.

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Discussion

BERGERON: In the small particle preparations from the dark-grown cells where you get pyridine nucleotide reduction if you add magnesium, is this reduction inhibited if substrates for phosphorylation are present as it is with the regular *Rhodospirillum rubrum* chromatophores?

FRENKEL: We have not worked with the small particle preparations.

ARNON: Dr. Frenkel said that *Rhodospirillum rubrum* would not grow on acetate in the dark but we have grown *Chromatium*, a photosynthetic sulphur bacterium, on acetate without added CO₂ or under conditions when CO₂, which might be formed from acetate, would be swept out by continuously bubbled gas. Under these conditions there would be enough CO₂ for it to act as a catalyst but not as a substrate. Apparently, there are differences between these two organisms. My second point concerns the reduction of pyridine nucleotides in the dark, i.e. a case when the photosynthetic process becomes limited to ATP formation or to what we call cyclic photophosphorylation. As we shall discuss this afternoon, *Chromatium* can use hydrogen gas to reduce pyridine nucleotides in the dark. In *Chromatium* supplied with hydrogen gas light is required only for ATP formation. If exogenous ATP is substituted for light, then *Chromatium*, which unlike *R. rubrum* is normally a strict phototroph, now becomes able to assimilate carbon dioxide in the dark.

FRENKEL: I was very surprised to hear that *Chromatium* can get along without CO₂. We have found that *Rhodospirillum*, when grown on standard media in the dark or light, has a definite CO₂ requirement which saturates at about 1 per cent CO₂. Thus far we have not been able to replace this CO₂ requirement by intermediates of the Krebs tricarboxylic acid cycle, or by a number of other well-defined chemicals. Only high concentrations of yeast extract or casein hydrolysate were effective in relieving this CO₂ requirement. With regard to Dr. Arnon's

second point it may not always be safe to generalize about the mechanism of photosynthesis from one special case. The observation that *Chromatium* can reduce pyridine nucleotides in the dark with molecular hydrogen, does not appear to preclude the possibility that *Chromatium* could reduce pyridine nucleotides directly or indirectly by a photochemically generated reductant.

ARNON: The point is that hydrogen gas has been known for almost 30 years as a physiological electron donor for *Chromatium*. The utilization of hydrogen gas by this organism is not to be regarded as an experimental artifact. We do not build but merely support our theory with the facts of *Chromatium* photosynthesis. We have presented other lines of evidence elsewhere.

FRENKEL: We have tried to grow *Rhodospirillum rubrum* with hydrogen but it does not grow very well.

ARNON: *Chromatium* grows very well with hydrogen gas.

BERGERON: Coming back to the question of acetate-grown *Chromatium* I should like to point out the truth in both points of view. Recently Dr. Benedict was trying to study carotenoid biosynthesis in *Chromatium* using labelled acetate, and he was using a medium containing a very small percentage of CO₂. They were growing very nicely. Some objection was raised to the carbonate so they were transferred to a medium which was identical except for the fact that the minimum amount of CO₂ was taken away. The new cultures grew slowly but finally got going again on the pure acetate.

ARNON: Let me make it clear again that when I say that *Chromatium* grows without CO₂, I do not imply that CO₂ is not used catalytically; I firmly believe it is. What I am saying is that we have grown *Chromatium* without any added supply of CO₂ and under conditions where any large concentrations of endogenous CO₂ would be swept out by bubbling gas.

FRENKEL: What is the pH of the medium?

ARNON: They grow at pH between 7 and 7.8.

FRENKEL: Under these conditions it may not be too simple to remove the CO₂ which is produced metabolically at a rate adequate to prevent its re-utilization.

ARNON: As I said earlier I firmly believe that the CO₂ is used catalytically.

FRENKEL: In studies on the effect of CO₂ on the growth of micro-organisms, experimental conditions are not always adequately described. At low gassing rates, with actively metabolizing cells a steady state concentration of CO₂ may be built-up permitting continued growth of the micro-organisms.

KAMEN: I should mention that in practically all the chloroplasts structures which are known there is a very high concentration of chlorophyll held to the protein. In the bacteria, as you may have noticed in Table 1 (p. 280), the ratio of chlorophyll to protein is about 15, whilst in the case of chloroplasts, it is something of the order of 1500. In the case of the nucleotides, also, the concentration of the nucleotides in chromatophores is about 1 to 15 chlorophylls, while in spinach chloroplasts it is over 1 to 2500 as Krogman showed, so there is a quantitative difference between structures producing oxygen and those which don't. I believe that this may have something to do with the Emerson effect which indicates that at least two quanta are funnelled to each active site to get the oxygen off. It is very difficult for an organism with limited amounts of chlorophyll to funnel the quanta to where it wants it.

VERNON: I can give you some information about the relation between phosphorylation and photoreduction reactions. In our laboratory we have followed photophosphorylation, the photoreduction of DPN and the photo-oxidation of ascorbate. In the experiments we have performed, there is a large degree of independence between these reactions. The photoreduction does not require an associated phosphorylation and the photophosphorylation does not require an associated photoreduction of DPN. This supports your idea that these two reactions are separate and distinct.

FRENKEL: I believe in your paper on pyridine nucleotide reduction you mentioned that ADP and ATP inhibited reduction. Did you find that simultaneous phosphorylation will inhibit the reduction?

VERNON: No, they are essentially independent.

ARNON: I would like to state that we now have some evidence for non-cyclic photophosphorylation in photosynthetic bacteria, that is coupled with the reduction of DPN. As in chloroplasts, non-cyclic photophosphorylation in bacteria, does not replace cyclic photophosphorylation but supplements it.

FRENKEL: In the work of Smith and Baltscheffsky light-induced phosphorylation by *Rhodospirillum* chromatophores was shown to be linked to the oxidation of cytochrome *c* and possibly also to the reduction of a *b*-type cytochrome.

ARNON: I am speaking of new evidence. It is perhaps premature to make the comment before the evidence is presented but I wish to make it now for the sake of completing the record of this discussion.

The Photosynthetic Macromolecules of *Chlorobium Thiosulfatophilum**

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There is considerable modern evidence that in higher plants the entire process of photosynthesis occurs in a microscopic but complex organelle—the chloroplast (see review by Arnon [4]). The study of simpler systems offers an advantage by eliminating variables which are not pertinent for analysis of the basic light-dependent phenomena. This consideration accounts, in large measure, for the current interest in bacterial photosynthesis.

Knowledge of the submicroscopic basis of bacterial photosynthesis is still fragmentary but is improving rapidly. It was assumed, until 1952, that the photosynthetic pigments are bound to protein and dispersed throughout the organism. At that time it was reported by Pardee *et al.* [33] that the pigments sediment rapidly in crude extracts of the non-sulphur purple bacterium, *Rhodospirillum rubrum*. The authors isolated the pigmented component and applied the name chromatophore to it. The electron micrographs of chromatophores which had been dried and shadowed with metal revealed disks about 1100 Å in diameter. It was inferred that the disks represented spheres with a diameter of about 600 Å. This value agreed roughly with the diameter of 400 Å which had been calculated by Stokes relation from the sedimentation coefficient (200 S) of the purified preparation [35]. At the same time, similar electron micrographs also were obtained by Thomas [38] with crude extracts of several photosynthetic bacteria. These reports provided concrete evidence that the pigments are localized in structures which are several orders of magnitude larger than soluble proteins.

The first evidence that this order of structural organization could be associated with a relatively high degree of functional capability was provided in the report by Frenkel [19] of the light-dependent phosphorylation of ADP by subcellular preparations of *R. rubrum*. This report and the

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description of photosynthetic phosphorylation in chloroplast preparations by Arnon *et al.* [2, 3] supplied direct evidence for the idea of Emerson *et al.* [17] that the role of light is to produce energy-rich bonds and also strengthened the belief that the fundamental photochemical events are the same in all photosynthetic organisms. Since that time considerable information has accumulated about various properties of pigmented preparations of photosynthetic bacteria (see papers in this symposium and review by Frenkel [21]). However, only two organisms have been studied in any real detail, *R. rubrum* and *Chromatium*, the purple sulphur bacterium. The chromatophore of *Chromatium* represents the simplest level of structural organization which is known to support photophosphorylation. We have considered the structure and function of this chromatophore in some detail previously [6, 7]. This system provides the perspective for a study which is in progress of the photochemical apparatus of the green sulphur bacterium, *Chlorobium thiosulfatophilum*. In the interest of clarity, the data, of several kinds, are considered at successive levels of organization; the organism, the crude extracts, and the purified pigmented component.

Results

THE ORGANISM

The green sulphur bacterium, *Chlorobium thiosulfatophilum*, is a strict anaerobe and an obligate phototroph. It can use hydrogen sulphide, thio-sulphate, tetrathionite, elementary sulphur, or molecular hydrogen as the electron donor for carbon dioxide assimilation [29]. The quantum requirement of the light dependent process is 8-10 quanta for four hydrogen atoms (or four electrons) moved with molecular hydrogen, thiosulphate or tetrathionite as the reducing agent [30]. This agreement with the value obtained with the other photosynthetic organisms implies a fundamental similarity in the basic light-dependent events (cf. [34]). In the laboratory the organism is cultured in an inorganic medium containing carbonate, sulphide and thiosulphate [31]. Depending upon growth conditions and age, the *in vivo* absorption spectrum shows differences due to variation in the content of accessory pigment (Fig. 1). All the data reported here are based upon cultures with a moderate amount of accessory pigment. It has recently been demonstrated [37] that contrary to previous assumptions, at least two different molecular species of chlorophyll exist among the green bacteria. The organism used in this study contains *Chlorobium* chlorophyll-650 and is strain L.

A representation of the submicroscopic morphology of this organism can be obtained by electron microscopy of ultra-thin sections. Typically, the organisms are fixed by exposure to osmium tetroxide at a concentration

of 2%, in a medium buffered at pH 7.4 and corresponding to the culture medium except for the omission of reducing substances. The specimens are dehydrated in a graded series of alcohols with the temperature progressively reduced to -50° , then the specimens are infiltrated with butyl methacrylate monomer and polymerized by gamma radiation from a cobalt-60 source at -50° . In sections through an axial plane (Fig. 2) the peripheral envelope, or cell wall, is distinct from the cytoplasmic membrane. The "nucleus" is represented by the axial region of low electron density which contains ramifying spiral filaments. Typically, there are one or more relatively large circular areas of high electron density which

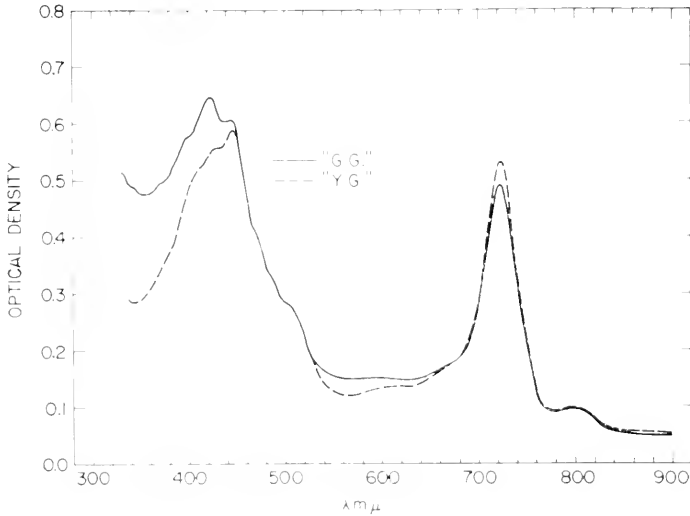


FIG. 1. Illustration of the range of variation in content of accessory pigments in cultures of *Chlorobium thiosulfatophilum*. The absorption spectra were measured (Cary Model 14) through opal glass to reduce the effect of light scattering.

represent sections through inclusions rich in polyphosphates. The cytoplasmic region has a stippled appearance owing to the presence of large numbers of small particles. The lack of contrast between the particles and the background prevents accurate determinations of size and form but it is clear that the images are circular rather than elongated and have a maximum extension of about 150 Å. There is no indication of the vesicular chromatophores (Fig. 3) which characterize *Chromatium* [6, 7, 39], *Rhodospirillum rubrum* [22, 25, 39], *Rhodospseudomonas spheroides* [39], and *Chlorobium limicola* [39] of the peripheral lamellae which have been or, described in *Rhodomicrobium vannielii* [40], or of the lamellated inclusions of *Rhodospirillum molischianum* [16]. In fact, the above description,

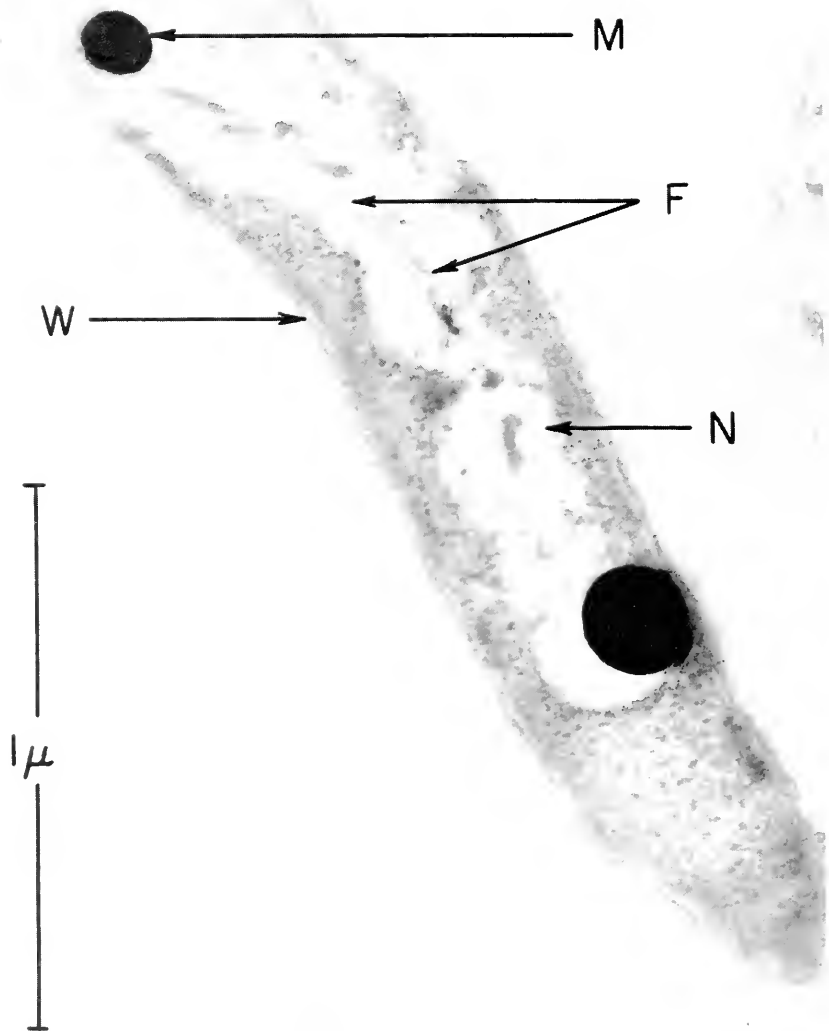


FIG. 2. Electron micrograph ($72\ 000\times$) of a thin section in the axial plane of *Chlorobium thiosulfatophilum*. The cell wall (W), which tends to appear as two layers, is distinct from the cytoplasmic boundary. The "nuclear" region has a low electron density and contains filaments (F) which ramify. Metaphosphate inclusions (M) appear as circular areas of high electron density. The cytoplasm is filled with small particles which have a maximum diameter of about $150\ \text{\AA}$.

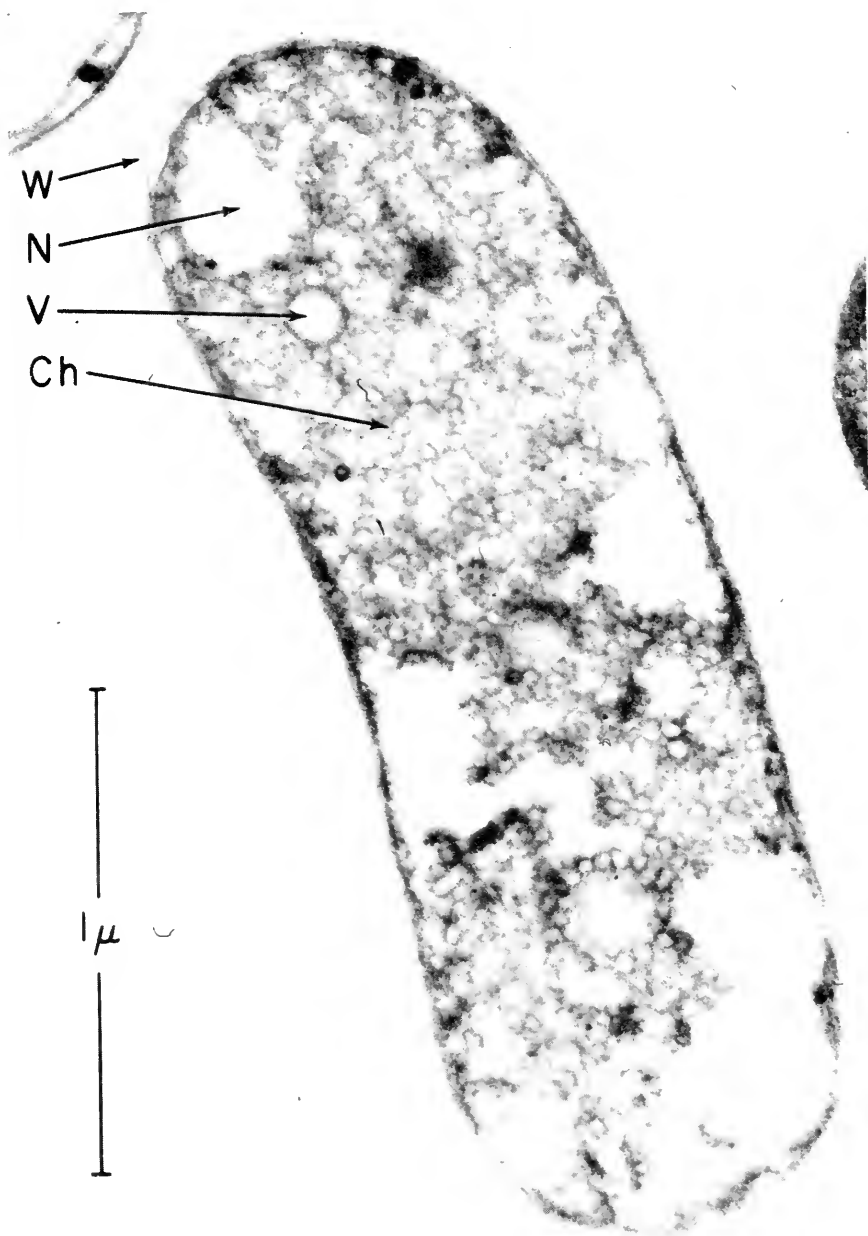


FIG. 3. Electron micrograph ($65\ 000\times$) of a thin section of the purple sulphur bacterium, *Chromatium*. The organism is smooth contoured and is bounded by a dual membrane which can separate into two distinct structures, the cell wall (W) and the plasma membrane. The cell is filled with the chromatophores (Ch) which are minute vesicles. These appear as annular images with an outer diameter of about $300\ \text{\AA}$ and a cortical thickness of about $70\ \text{\AA}$. Large vesicles (V) about $1000\ \text{\AA}$ in diameter are also visible. The irregular areas of low density (N) are considered to be parts of an irregularly shaped nuclear compartment. The closely packed chromatophores conceal the small particles (Sp) which are observed in the fractions.

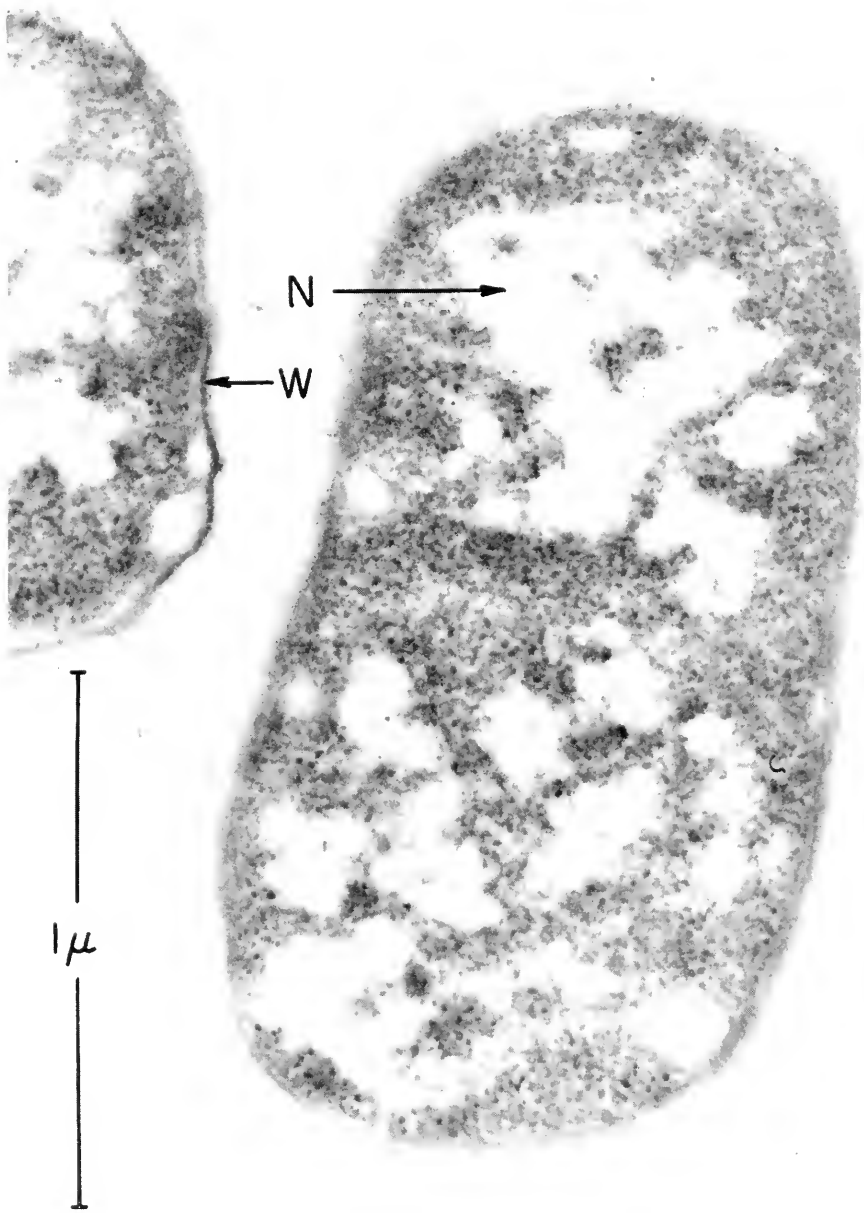


FIG. 4. Electron micrograph ($72\ 000\times$) of thin sections of the non-photosynthetic bacterium *Escherichia coli*. The cell wall (W) is distinct in suitably oriented sections. The "nuclear" region (N) has a low electron density and contains very fine ($\sim 40\text{ \AA}$) filaments. The cytoplasmic region contains minute particles which are well defined.

excepting the inclusions, could apply to typical non-photosynthetic bacteria [9, 11, 12, 13, 28, 32] such as *Escherichia coli* (Fig. 4).

CRUDE EXTRACTS

It is rather difficult to rupture this organism but two methods have been used successfully: breaking frozen cells in the Hughes press or exposing a suspension of 2 g. of cells (wet weight) and 1 g. of very fine synthetic sapphire abrasive (Linde B) in 40 ml. of 0.1 M tris (hydroxymethylamino methane) buffer at pH 7.8 to sonic oscillation for 2 min. at $0-5^{\circ}$ in a 10 K.C. Raytheon oscillator.

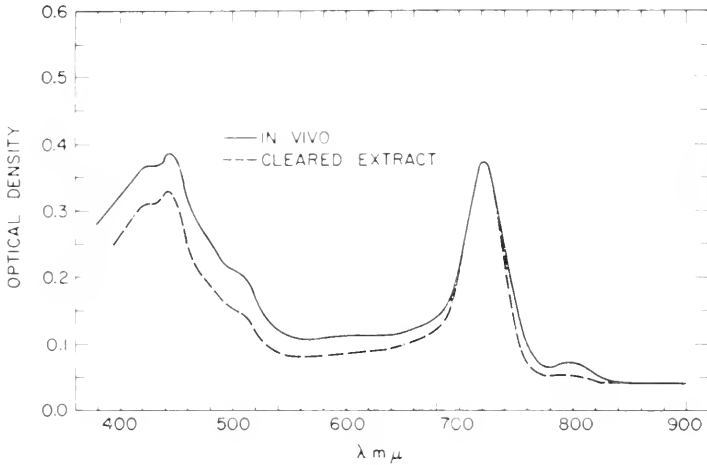


FIG. 5. Illustration of the correspondence between the absorption spectrum of the photosynthetic pigments *in vivo* and in the cell-free extracts. The divergence at lower wavelengths is attributable to differences in light scattering which were not completely compensated by the use of opal glass.

Cells and debris are removed from the crude extract by two successive centrifugations for 30 min. with refrigeration (5) at 26 000 g. The "cleared" extract has an absorption spectrum which corresponds with the *in vivo* spectrum (Fig. 5). The small differences which are observed are attributable to differences in light scattering. This agreement provides some assurance that the physicochemical characteristics of the pigment bearer have not been disturbed greatly during the process of cell disintegration.

A pigmented fraction, free from other macromolecular constituents, can be prepared by repetitive centrifugation for 2 hr. at 144 000 g under refrigeration (5). The progress of the fractionation is indicated by changes in the components observed in the analytical ultracentrifuge and by

changes in the absorption spectrum (Fig. 6). The sedimentation diagram of the "cleared" extract reveals three major components with sedimentation coefficients of about 5, 30, and 50 Svedberg units (S) respectively. The colour due to the photosynthetic pigments is related to the most rapidly sedimenting component. It is easy to eliminate the slowest component (5 S) by repetitive centrifugation but it is rather difficult to elimin-

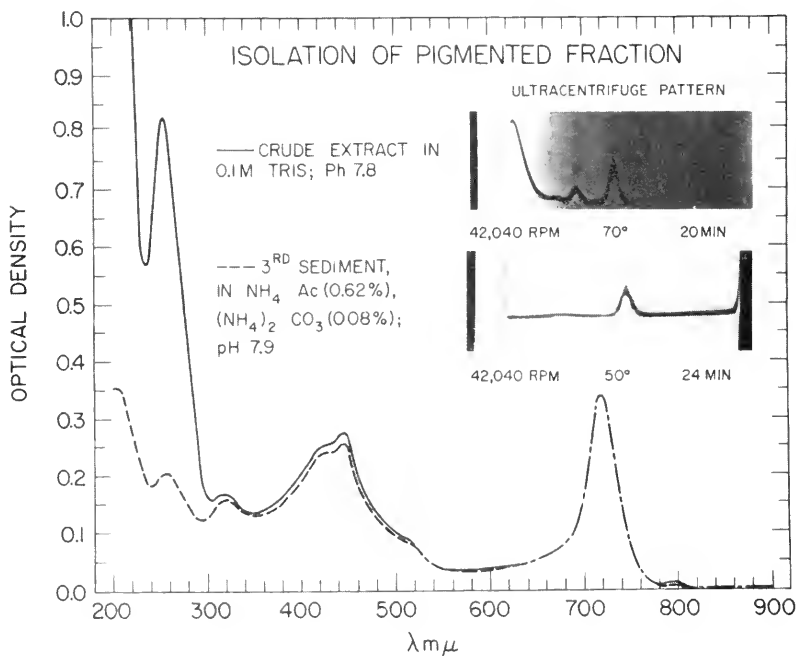


FIG. 6. Record of the changes in the sedimentation diagram (Spinco Model E) and absorption spectrum (Cary Model 14) during the isolation of the pigmented component. There are three major components in the crude extract. The colour due to the photosynthetic pigments is associated with the component (50 S) which sediments the most rapidly. The absorption at 260 $m\mu$ due to nucleic acid is greatly reduced as the slower components are eliminated.

ate completely the 30 S component under conditions which recover the pigmented component (50 S) in high yield. The progressive elimination of the two slower components is reflected in the absorption spectrum by the drastic reduction in nucleic acid absorption at 260 $m\mu$.

If a crude extract is placed upon a linear sucrose gradient (10% to 50%) and centrifuged for several hours in a swinging bucket head at 156 000 g two coloured zones develop, the "green" zone with a sharply defined leading edge is followed by a less distinct "yellow" zone. Com-

parison of the absorption spectra of these zones with the original extract reveals (Fig. 7) that the "yellow" zone is rich in carotenoids and deficient in chlorophyll. The "green" zone, which accounts for the bulk of the material, shows an appreciable decrease in the absorption due to carotenoids. Two alternative explanations of this phenomenon are either that a considerable fraction of the intracellular carotenoids are not incorporated into the pigmented particle or that these pigments are exposed and tend to strip off during the progressive movement into more concentrated

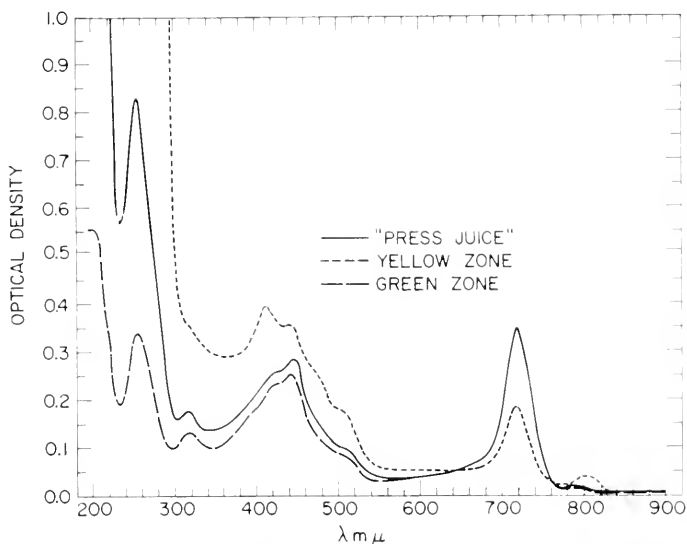


FIG. 7. Effect of centrifuging the crude extract in a linear gradient of sucrose (10%–50%). The pigmented particles are recovered in a "green" zone which is followed by a "yellow" zone. By comparison with the original absorption spectrum, the "yellow" zone is enriched in carotenoids and deficient in chlorophyll. The "green" zone shows a decline in the absorption due to carotenoids and nucleic acids.

sucrose solutions. The latter alternative seems to be favoured by the observation that these pigments are not left in the original layer but are recovered in the sucrose gradient behind the green zone; then too, this tendency is well defined only when gradients are employed. The spectra also show disturbances in the 800 $m\mu$ maximum which are reminiscent of the effect of carotenoids upon the infra-red absorption spectrum in *Chromatium* [1, 6, 8, 23, 24].

The components present in these extracts resemble in number and sedimentation characteristics the extracts of non-photosynthetic bacteria. In the photosynthetic bacteria which have been investigated previously [35] the chromatophores have been present as a component in addition to

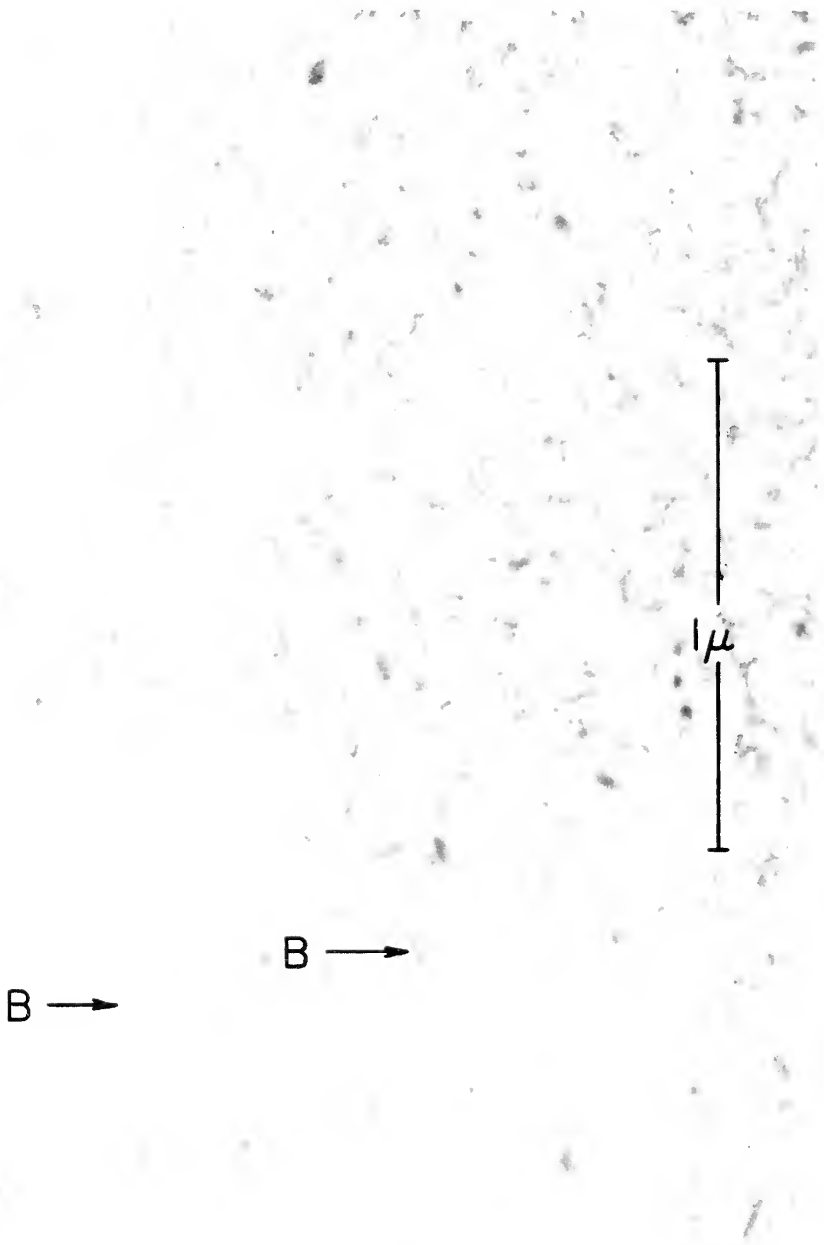


FIG. 8. Electron micrograph (72 000 \times) of a thin section of a pellet of a pigmented fraction which sedimented previously as one component (50 S) in the analytical ultracentrifuge. The preparation consists predominantly of small particles with a diameter of about 150 Å . There are also occasional elongated figures (B) which are either contaminants or aggregates.

three such slower components. Thus, the resemblance of *Chlorobium thiosulfatophilum* to *E. coli* which was observed in the electron micrographs is carried over into the components present in extracts. The obvious distinction is in composition. In these extracts the 50 S component bears photosynthetic pigments and appears to be free of nucleic acids.

THE PURIFIED PIGMENTED COMPONENT

Characterization of the physicochemical properties, composition and photochemical activity of the pigmented component is incomplete but suffices to place limits on a number of pertinent variables. We have two indices of the homogeneity of the fraction; sedimentation behaviour and electron microscopical observation. Although refined analysis may reveal complexities, it is clear that the photosynthetic pigments do sediment with one component which exhibits a well-defined spike. To this extent, the preparation appears to be monodisperse with regard to the photosynthetic pigment. When the pellet of a pigmented fraction that sedimented essentially as a single component is removed from the analytical ultracentrifuge and processed for electron microscopy, the thin sections (Fig. 8) reveal a rather uniform population of particles. These particles resemble the particles seen in the cytoplasm of the cell; that is, the maximum extension of the image in any direction is about 150 Å. If these particles are slightly elongated the range of deviation is probably between 100 and 150 Å. The sedimentation of the pigmented fraction depends to a considerable degree upon concentration and the data are still inadequate for an accurate calculation of the sedimentation constant; however, the maximum value obtained from the purest preparations at high dilution is 50 Svedberg units when converted to 20° in water. It is of interest to compare the direct and indirect data on particle size and obtain some idea of the agreement. If we use a sedimentation constant of 50 S and the conventional assumption of a density of about 1.2 g./ml., then the particle diameter calculated from Stokes relation is 173 Å. This degree of agreement with the electron microscopical observation is reassuring. A spherical particle of this size and density would have a molecular weight between 1.3 and 1.6 million. This value can be used as a first estimate of the molecular weight.

An interesting but troublesome property of the 50 S particle is the tendency to aggregate into a series of more rapidly sedimenting components as the degree of purification becomes relatively high. Electron microscopic examination of thin sections of pellets of these aggregates (Fig. 9) reveal elongated profiles with diameters up to 400 Å and lengths of thousands of Å. When such preparations are sprayed upon on specimen supports and shadowed with metal the electron micrographs reveal rigid

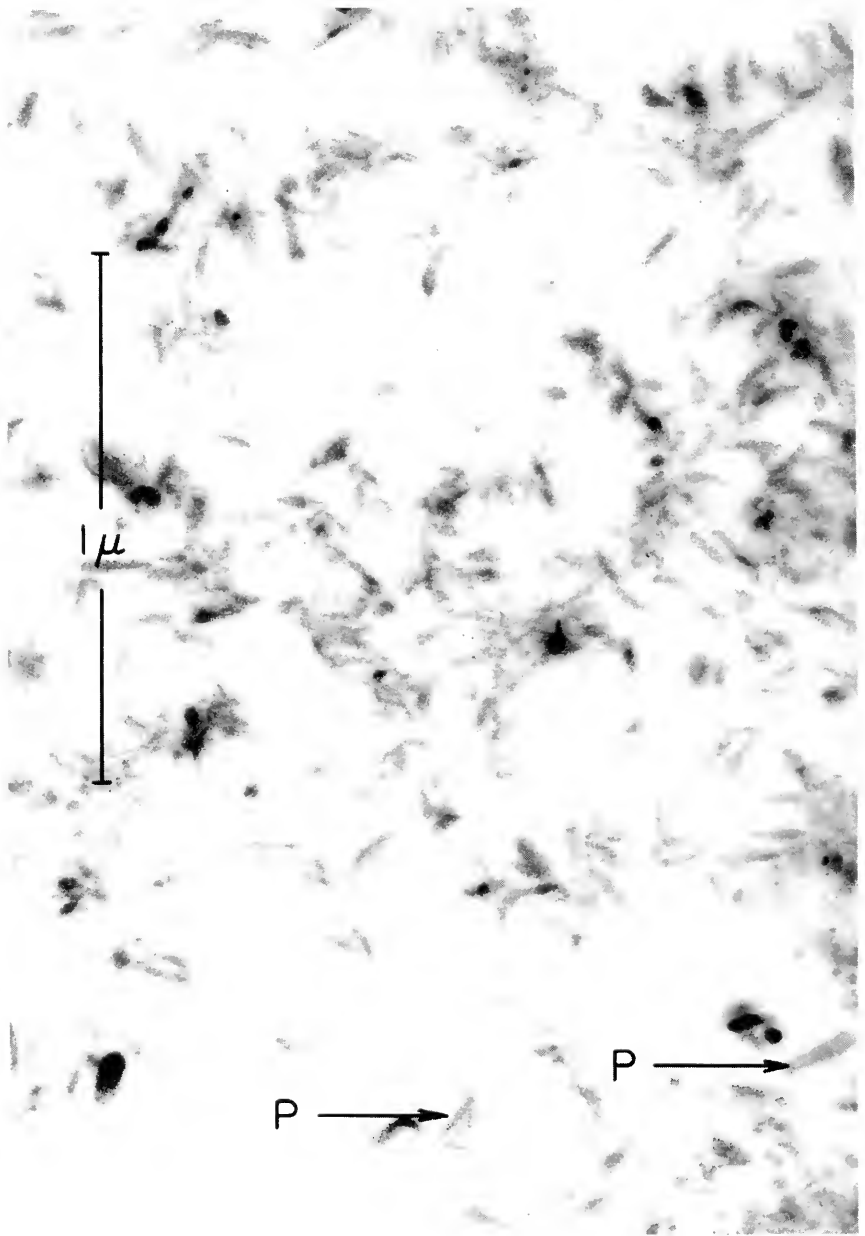


FIG. 9. Electron micrograph ($72\ 000\times$) of a pigmented fraction which changed into a series of more rapidly sedimenting components during purification. There is a preponderance of elongated figures which tend to pair (P). The size is not uniform. The widths appear to be multiples of a minimum value of about $150\ \text{\AA}$. The length also varies but cannot be established in thin sections. The maximum values observed are in the $1000\ \text{\AA}$ range.

rods. This tendency of the macromolecules to organize into structures with a higher degree of order than is observed in the organism is unusual.

In the initial phase of this study, observations on the chemical composition of the pigmented fractions have dealt primarily with chlorophyll, protein and nucleic acids. The spectrophotometric assay of chlorophyll content is based on the specific absorption coefficient of this chlorophyll in acetone [37] and a recent estimate of the molecular extinction coefficient [14]. The quantitative relationship between the 723 m μ maximum in the *in vivo* spectrum and the 652 m μ maximum of the chlorophyll in acetone has been determined in order to allow direct measurement of chlorophyll

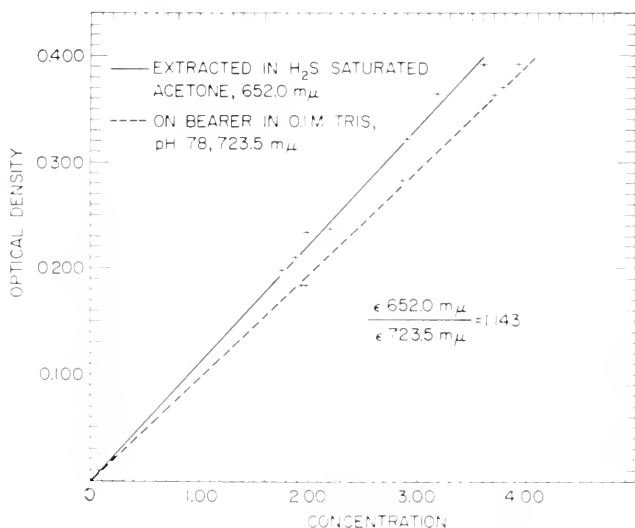


FIG. 10. Quantitative determination of the relationship of the absorption at 652 m μ of the chlorophyll in H₂S-saturated acetone and the absorption at 723.5 m μ of the chlorophyll bound to the pigmented particle.

content in the extract during fractionation (Figs. 10 and 11). For the same reason the nucleic acid and protein estimates have been limited to ultra-violet absorption measurements. During the course of fractionation the absorption maximum at 260 m μ decreases from an initial value which is two or three times greater than the chlorophyll maximum at 723 m μ to a limiting value which is 1/3 of the chlorophyll absorption. The initial 280/260 m μ absorption ratio is about 0.5 and increases to 0.9; thus, the 50 S fraction is quite free of nucleic acids. On a mass basis, the chlorophyll:protein ratio in the more highly purified preparations is 1/13. Assuming that these two components account for the bulk of the mass of the particle, this ratio represents about 100 chlorophyll molecules for each particle with a molecular weight of 1.5 million. The cytochromes present in the

purified fraction have been studied in some detail [26]. On the basis of the above estimate of particle size, the cytochrome content approaches limiting proportions, that is, 1 or 2 cytochromes per particle.

Several years ago it was reported by Williams [41] that photophosphorylation might have occurred in experiments with crude extracts of a green sulphur bacterium. The observations in our laboratory indicate that light-dependent uptake of phosphate occurs in the "cleared" extract but disappears or is greatly reduced during the course of fractionation. Further

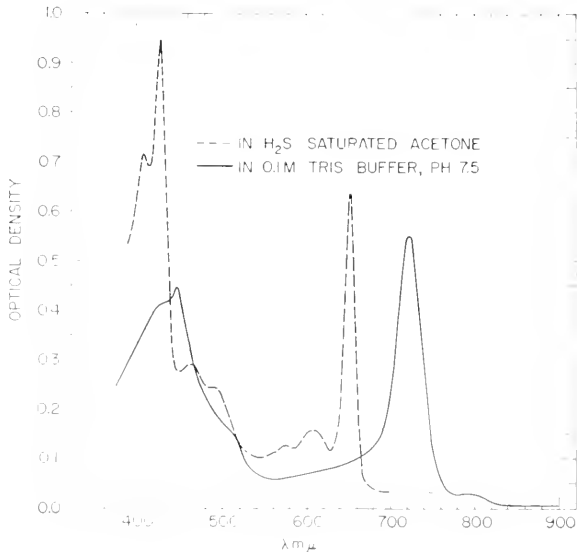


FIG. 11. Quantitative comparison of the spectral change produced by transferring the photosynthetic pigments from the physiological environment of the pigmented particle into solution in H_2S -saturated acetone.

work is needed to establish whether or not the phosphate uptake in the crude extracts really represents ATP formation and what relationship the particles have to this activity.

Discussion

The wide range of opinion which exists concerning the importance of structural organization for photosynthesis is illustrated in a recent symposium entitled "The Photochemical Apparatus—Its Structure and Function" (*Brookhaven Symp. Biol.* 11, 1958). Structural organization above the molecular level was regarded as essential, or important for efficiency, or irrelevant depending upon the type of data and particular aspect of photosynthesis under consideration. Most of the participants,

however, were prepared to assume that structural organization probably contributes to efficiency and might even be essential for the primary process. Those so inclined inferred that the submicroscopic lamella, present in rudimentary form as the cortex of the chromatophore and elaborated in the grana and stroma of the chloroplast, is a universal architectural characteristic of photosynthetic systems. Since the properties of a lamella fulfill the requirements for the separation of primary reducing and oxidizing components in a photolysis scheme as well as for the condensed state required in the semiconductor concept of the primary photosynthetic process, a theoretical basis could be proposed to account for the universality of this characteristic.

Our approach to the problem has been to try to characterize both structure and function at the lowest level of structural organization capable of supporting photosynthesis. The present study of the structure and function of the photochemical apparatus of *Chlorobium thiosulfatophilum* is raising some questions. The data lead tentatively to the following concept. The structural unit is a particle which is spherical rather than elongated, has a maximum dimension of about 150 Å and a molecular weight of about 1.5 million. Such a macromolecule is about one order of magnitude smaller in mass than the simplest photosynthetic unit studied to date; namely, the *Chromatium* chromatophore. Does this particle represent the limiting size of chromatophore or a lower level of organization? Although it is possible to construct a sphere 150 Å in diameter with a 60 Å thick cortex, the area available at the inner surface could accommodate only about one-tenth of the chlorophyll contained in the particle; thus, the type of architecture postulated for the *Chromatium* chromatophore [6] is not applicable to this system. In addition, in some properties, the pigmented particle differs rather sharply from the chromatophores which have been studied; for example, in the degree to which accessory pigments separate from the particle and the tendency of the particles to aggregate into progressively larger rod-like structures. For these reasons, we prefer, for the time being, to regard this system as less highly organized than the chromatophore and refer to the pigmented component either as photosynthin, a name employed for the pigmented extracts prior to the advent of the chromatophore concept [18] or as holochrome, the term used to designate "a colored substance as it exists in its natural state within an organism, where the colored group is combined or associated with a carrier which alters the physical or physiological properties of the prosthetic group" [36].

If we assume that the lamella is not an architectural feature in this system, then the idea that lamellar organization is essential for photosynthesis is brought into question. One solution to this dilemma is to consider the lamella as the expression of a more fundamental characteristic

which can be extrapolated to the molecular level. An application of this concept is illustrated in the model proposed for the ultrastructure of the *Chromatium* chromatophore [6]. In this instance (Fig. 12), the submicroscopic architecture is generated by the juxtaposition of molecular units. As in crystallization, it is the properties of the building block, a pigmented protein with hydrophilic and hydrophobic poles, which determine the

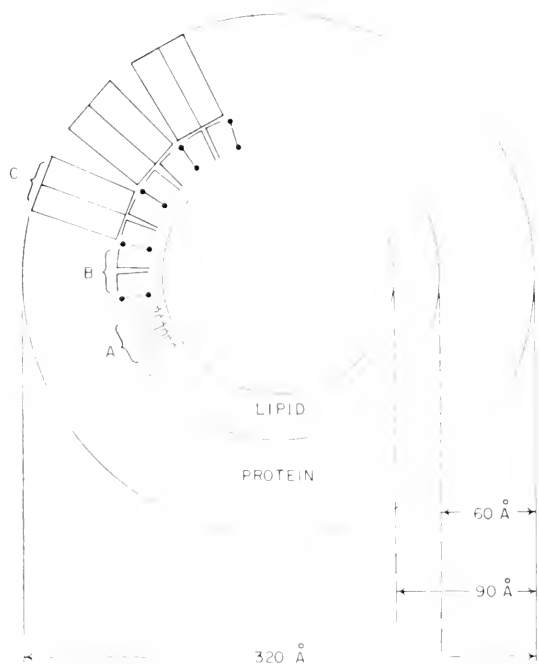


FIG. 12. Working hypothesis of the ultrastructure of the chromatophore. The *Chromatium* chromatophore is described as a hollow sphere about 320 Å in diameter with a cortex about 90 Å thick. The pigment molecules (B) aligned in a monolayer are bounded internally by a phospholipid (A) monolayer and externally by a 60 Å thick protein layer. The "minimal unit" of composition has been used as a structural subunit. The protein has been folded and is related directly to two chlorophyll molecules. On the average the protein is related indirectly to one carotenoid molecule and ten phospholipid molecules.

form of the assemblage. Such a scheme provides for specificity in a protein of conventional dimensions, requires no assumptions beyond the principles of molecular interaction for obtaining a higher level of organization, and also allows for great flexibility in the composition of the lipid phase.

If structural organization, above the molecular level, is a prerequisite for photosynthesis, then the *Chlorobium* holochrome must approach the limiting conditions. This particle is already within the physical range of

materials, such as the haemocyanins, which are classed as respiratory proteins. Such considerations as these add interest to the study of the photochemical activity and composition of this holochrome.

Unequivocal evidence of photophosphorylation has not been obtained with the isolated holochrome, but many reasons can be advanced to explain failure. There is no indication, so far, in the composition data that this particle is fundamentally different from the other photosynthetic systems. There are two pigment types, the chlorophyll and the carotenoids. There are enough chlorophyll molecules present [100] to meet the requirements of the semiconductor hypothesis. The cytochromes, which figure prominently in current concepts of the primary events [5, 27] are present albeit in near-limiting quantities. It is not known, however, whether the important [10, 15] lipid-soluble quinone compounds are present.

It seems reasonable to expect that continued study of this system will help to define fundamental relationships between structure and function in photosynthesis.

Acknowledgments

We wish to thank Dr. S. Conti and Miss H. Kelly for culturing the organisms employed in this study. The skilful technical assistance of M. Gettner and W. Geisbusch is also gratefully acknowledged.

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Discussion

CHANCE: Was there more than one kind of cytochrome present in the purified particles?

BERGERON: I am not speaking from my own data now, I am speaking for Hulcher and Conti [26]; there appears to be an *f*-type and an *a*-type.

Some Physical and Chemical Properties of the Protochlorophyll Holochrome

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Protochlorophyll, the chlorophyll precursor of dark-grown seedlings, has been isolated in active form. Even though this material is separated from the plant it is transformed by light to chlorophyll. Since the active material is proteinaceous, it can be extracted and fractionated by the procedures of protein chemistry. The active material is particulate and shows a distinct sedimentation peak in the ultracentrifuge diagram [1] with sedimentation constant of about 16 to 17 Svedberg units. If it is assumed to have the same density, 1.33, as many other proteins with the same sedimentation constant, its molecular weight would be about 400 000. Later experiments have shown the particle to have a density in solution of approximately 1.16. Based on this density the molecular weight would be about 700 000 [2]. This agrees fairly well with a molecular weight of 900 000 calculated from the ratio of protochlorophyll to protein obtained by analysis [2] when a molecular ratio of one, for pigment to protein, was assumed. Conversely, the agreement of the results obtained by analysis and by centrifugation supports the assumption that the pigment-protein complex contains only one protochlorophyll component.

Carotenoids in protochlorophyll holochrome

Although the centrifugation pattern indicates a fairly homogeneous molecular species in respect to molecular weight, the carotenoid content of the isolated material shows that all the particles cannot be of exactly the same composition. The carotenoid-protochlorophyll ratio varies from preparation to preparation. Furthermore, on occasion, the molecular ratio of total carotenoid to protochlorophyll is about one-half [3]. If each holochrome particle contains one protochlorophyll, then some particles lack carotenoids entirely. But the carotenoid fraction is made up of several carotenoids, as reference to Fig. 1 shows, and this complicates the stoichiometry of the holochrome still further.

The ratios of carotenoid to protochlorophyll imply either of two

possible situations. One is that on any individual holochrome particle no fixed ratio of yellow pigments to protochlorophyll exists. This means, therefore, that the holochrome cannot be a definite compound. The other situation is that lack in uniformity of composition results from contamination of the protochlorophyll holochrome by carotenoid holochromes. If this is true, the carotenoid and protochlorophyll holochromes must have very similar sedimentation and precipitation properties.

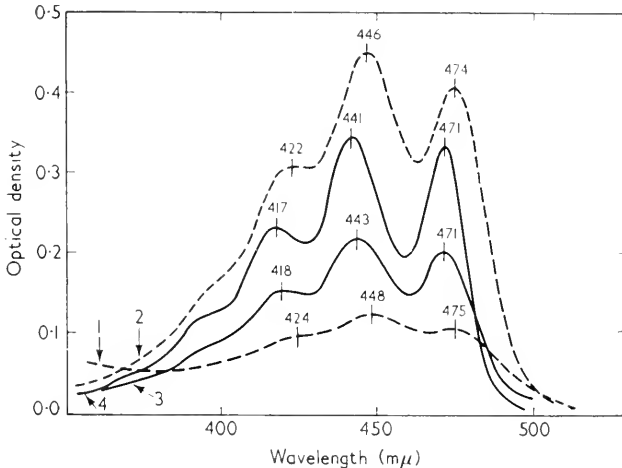


FIG. 1. Absorption spectra of carotenoid fractions from protochlorophyll holochrome isolated by chromatography on columns of powdered cellulose.

The carotenoids were extracted from the pigment-protein complex, named protochlorophyll holochrome, with 80% acetone. They were then transferred to petroleum ether and chromatographed on powdered cellulose. The chromatograms were developed by various mixtures of acetone in petroleum ether.

Positive identification of the individual carotenoids has not been made, but from the shapes of the absorption curves, the positions of the maxima, and certain colour reactions the pigments have been tentatively identified as lutein epoxide ester (curve 1), lutein (curve 2), isolutein (curve 3), and violaxanthin *b* (curve 4). These assignments differ from those given the carotenoids from etiolated bean leaves (*Phaseolus vulgaris*) by Goodwin and Phagpolngarm [4] who have identified the following: β -carotene, 10.8% of the carotenoid pigments present, lutein, 38.4%, neoxanthin, 50.7%, and a trace of an unknown yellow pigment. At present it is impossible to evaluate the cause of the discrepancies.

The function of the carotenoids in the holochrome is still unknown. Previously, it was intimated that these pigments played an obscure role in

the protochlorophyll conversion since the amount of chlorophyll formed by illumination was found to be statistically related molecule-for-molecule to the carotenoid present [3]. Now that we have found the carotenoid fraction to be made up of several constituents it would be necessary to assume that each constituent was equally effective in the conversion of protochlorophyll in order to account for the stoichiometry of the reaction. This is very unlikely, and militates against such an hypothesis. A further argument against such an assumption is that in etiolated albino leaves nearly complete conversion occurs in the absence of carotenoids [3]. It is improbable, therefore, that carotenoids are involved in the protochlorophyll transformation.

It has frequently been proposed that carotenoids function as inhibitors of the photo-oxidation of chlorophyll. The isolated carotenoid-containing holochrome, however, loses its chlorophyll by extended illumination. In this system, little protection against bleaching is afforded by the presence of yellow pigments.

Fluorescence polarization of protochlorophyll holochrome

When the fluorescence of a molecule is excited with plane polarized monochromatic light, the fluorescence emitted under certain circumstances may also be partly polarized. In principle, if a fluorescing molecule remains stationary and retains its absorbed energy during the interval between absorption of the exciting light and emission of the fluorescence, it emits fluorescence having a certain maximum degree of polarization. Polarization values lower than this indicate that the molecule has either rotated or else transferred its energy to like molecules during the period of excitation [5, 6]. Much can be learned about the state of fluorescent substances from measurement of this property. Because of this, the fluorescent properties of the protochlorophyll holochrome have been studied by this technique [7, 8].

The fluorescence polarization of the protochlorophyll holochrome was measured in an apparatus similar to that used previously by Goedheer [5]. To test the operation of the apparatus, the fluorescence polarization of chlorophyll in castor oil was determined. The value, 28.9, was found which agrees well with Goedheer's former measurement. *viz.* 28. Light from the cadmium arc, wavelength $644\text{ m}\mu$, was used for exciting the fluorescence.

Preliminary experiments in collaboration with Dr. Paul Latimer [7] gave polarization of fluorescence values for protochlorophyll holochrome lower than for chlorophyll in castor oil. This observation was corroborated by Goedheer and Smith [9], who obtained a value of 15 for a glycerine extract of protochlorophyll holochrome from etiolated bean leaves, and

18 for a glycine buffer extract (pH 9.5). Because of the irradiation necessary for measuring fluorescence polarization, the protochlorophyll is largely converted into chlorophyll, consequently, the fluorescence obtained is mostly from chlorophyll. These values, 15 and 18, are lower than the value for chlorophyll in castor oil, 28.9. Three possibilities suggest themselves to account for the lowered polarization: one, that the holochrome rotates more freely than chlorophyll immobilized in castor oil; two, that it transfers its energy to other chlorophyll molecules; or three, that the pigment is free to rotate within the holochrome.

Because the holochrome is so large, it cannot conceivably rotate fast enough to depolarize its fluorescence. The second suggestion of energy

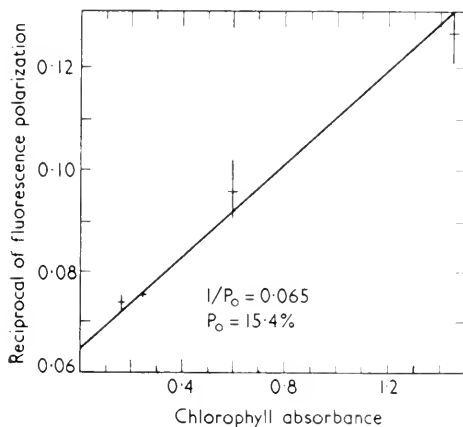


FIG. 2. The reciprocal of the fluorescence polarization of the protochlorophyll-chlorophyll holochrome plotted against the optical density of the chlorophyll maximum ($\sim 670 \text{ m}\mu$) at different stages of greening.

transfer between chlorophyll molecules also seems improbable in view of the small number of pigment molecules per holochromatic particle. This leaves only the third alternative as likely.

An estimate of the limiting fluorescence polarization value when no energy transfer exists can be obtained by extrapolating the fluorescence to zero pigment concentration. This was done by extracting the holochrome from leaves at different stages of greening, and by relating the fluorescence polarization with chlorophyll content through the expression

$$1/P = 1/P_0 + AC\tau \quad (8)$$

in which P is the polarization of fluorescence measured; A is a constant; C is the optical density of the chlorophyll peak at about $670 \text{ m}\mu$, which is proportional to the chlorophyll content; τ is the lifetime of the activated state; and P_0 is the polarization when C is zero. A plot of $1/P$ against

chlorophyll absorbance, Fig. 2, gave a straight line within the experimental error which extrapolated to a value of 15.4 for P_0 . This is in good agreement with the value of 15.0 obtained by direct measurement on the protochlorophyll holochrome. This indicates that the lower value of fluorescence polarization in the original protochlorophyll holochromes is not due to energy transfer between chlorophyllous pigments.

The third alternative put forward to account for the definite but sub-maximal polarization of the holochromatic pigment is that the pigment exists in the holochrome in such a way as to have partial freedom of rotation. This could be accomplished if the pigment were attached to the amino-acid "tails" of the holochrome protein similarly to haem in haemoglobin and myoglobin [8, 10].

Alkaline inhibition of protochlorophyll transformation

If the binding of haem in haemoglobin and of protochlorophyll in its holochrome are analogous, then the bonding of protochlorophyll to protein in the holochrome should be influenced by treatment with alkali at specific pH values [11]. The effectiveness of the various pH values for disrupting the pigment-protein complex should depend upon the acid dissociation constants of the amino-acid groups binding the pigment.

Inasmuch as the transformation of protochlorophyll to chlorophyll is stopped when the protochlorophyll is separated from the protein, a dissociation by treatment with alkali should stop the transformation. The pH at which the transformation is stopped should be characteristic of the ionization constant of the protochlorophyll-amino acid complex involved. Conversely, the pH values at which the transformation is stopped should indicate what amino acid groups hold the pigment. For this reason, a detailed study of the effect of alkali on the protochlorophyll holochrome and on the inhibition of the transformation has been undertaken.

Effect of pH on the protochlorophyll-chlorophyll transformation

The various degrees of inhibition of the protochlorophyll-chlorophyll conversion caused by treatment of the protochlorophyll holochrome at different alkalinities are shown in Fig. 3. The protochlorophyll holochrome was suspended in solutions of various pH values for different lengths of time. At stated intervals, samples were removed, neutralized with glycine, and spectrophotometered before and after being illuminated for 3 min. The optical densities of the chlorophyll formed were measured at the chlorophyll absorption maximum, $\sim 678 m\mu$. In Fig. 3, they are plotted as ordinate against the time of standing in the alkaline medium.

As is evident from Fig. 3, pH values between 7.20 and 9.16 have little

if any effect on the transformation. At pH 9.70, the alkalinity partly inhibits the transformation and as the alkalinity is increased to 10.27, 10.88, and 11.92 the inhibition is intensified. In order to estimate the maximum degree of inhibition at each pH, an algebraic equation was sought which permitted the limit of the transformation to be calculated from the experimental data. The third order reaction velocity equation did this as the concordance between experimental points, marked with circles, and calculated values, depicted by solid lines, demonstrates.

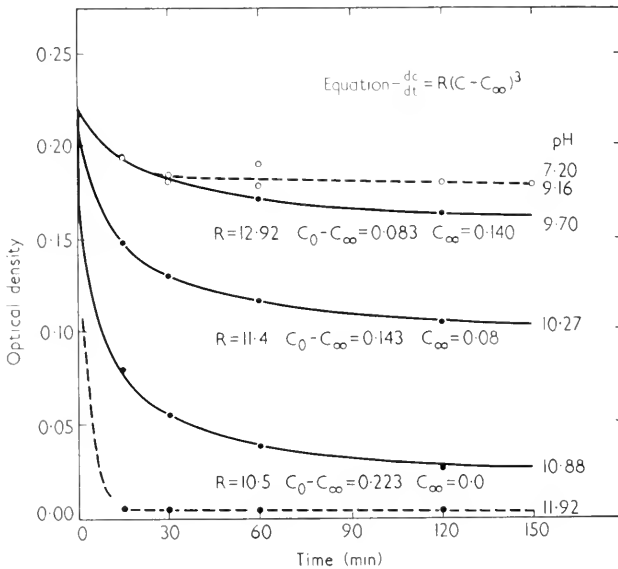
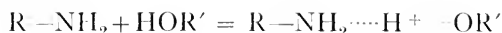


FIG. 3. Comparison of the rates of inactivation and limits of the transformation of protochlorophyll holochrome to chlorophyll holochrome at various alkalinities.

Between pH 9.70 and 10.88, the limiting value, C_{cc} , of the chlorophyll formed decreases with increase in pH. The rate constants, R , do not increase markedly in this range. The amount of pigment that can be inhibited from transforming at each pH, C_0 , increases with increase in pH. It is this increase of C_0 , rather than the change in the velocity constant, R , that causes the greater initial velocity of inhibition at higher pH. In fact, the initial velocity of inhibition at 9.70, 10.27, and 10.88 are directly proportional to the hydroxyl ion concentrations. At higher pH values the increased rate of inactivation must be due to a greater velocity constant rather than to an increase of inactivatable material, which has already reached its limit at 10.88.

The facts presented in Fig. 3 indicate that the inactivation reaches different limits depending on pH. One interpretation of this result is that

the pigment attaches itself to protein through a bonding that is sensitive to hydroxyl ions, such as amino groups that form ammonium compounds, or phenolic groups that act through hydrogen bonding. This may be illustrated as follows:



When the acidic hydrogen ion is neutralized the addition compound dissociates



In the case of the protochlorophyll-protein complex, when it is dissociated by hydroxyl ions, the protochlorophyll could no longer be transformed to chlorophyll by light. This is the proposed explanation for the inhibition of the transformation by action of hydroxyl ions.

The pH values that inhibit the photochemical transformation correspond to the dissociation constants of certain amino acids which may be implicated in the bonding of protochlorophyll. These amino acids with their approximate pK values [12] are ϵ -amino of lysine, 9.4 to 10.6, phenolic hydroxyl of tyrosine, 9.8 to 10.4, and the sulphhydryl of cysteine, 9.1 to 10.8. These values may differ considerably from one protein to another, and even in the same protein. For example, Stracher [13] found in the spectrophotometric titration of myosin two groups of tyrosine residues with pK values of 10.5 and 12.2 respectively. For this reason, no precise values for the pK values of the amino acids in the protochlorophyll holochrome can be assigned *a priori*.

IONIZATION CONSTANTS FROM TITRATION CURVES

The titration curve of protochlorophyll holochrome is shown in Fig. 4. From this curve it is obvious that two titration steps exist within the pH range effectively inhibiting transformation of protochlorophyll. The inflection points are at pH values of about 10.2 and 11.3. For groups with these pK values, ionization would be about 100% complete at pH 9.2 and 10.3. Thus it appears that the coincidence of the pK values from the titration curve and the pH values effectively inhibiting protochlorophyll conversion makes the assumption reasonable that the pigment is bound to protein through the amino acid residues—the most likely candidates being lysine, cysteine, and tyrosine.

IONIZATION CONSTANTS FROM SPECTROSCOPIC MEASUREMENTS

Changes in pH profoundly modify the protein part of the protochlorophyll holochrome as variations of the ultraviolet absorption spectrum show. In Fig. 5 is pictured the absorption of protochlorophyll holochrome

in the visible and ultraviolet regions of the spectrum. The absorption in the ultraviolet is due very largely to the protein part of the holochrome. In Fig. 6 is shown the effect of pH on the ultraviolet absorption of the

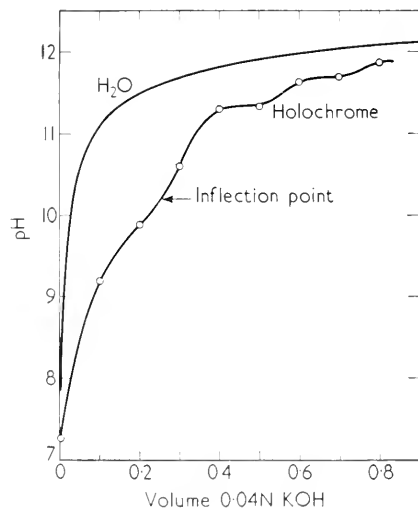


FIG. 4. The alkaline titration curve of protochlorophyll holochrome.

holochrome. At about pH 9.7, spectral changes become obvious. Perhaps it is noteworthy that at this alkalinity the inhibition of the protochlorophyll conversion begins to intensify significantly.

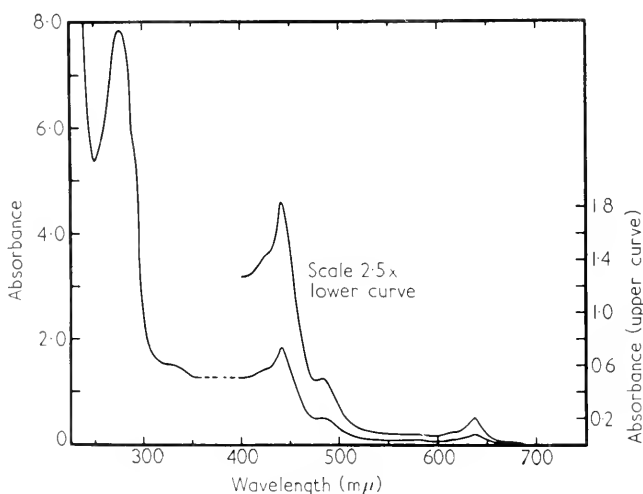


FIG. 5. The absorbance of protochlorophyll holochrome in the visible and ultraviolet regions of the spectrum.

From the changes in absorption with changes in pH it is possible to calculate pK values for the components undergoing change. The equation for this calculation is

$$\text{pK} = \text{pH} - \log A + \log (A_0 - A)$$

Here pK and pH have their usual meaning, and A is the absorbance change at a particular pH while A_0 is the maximum change in absorbance produced by increase of pH. The change in absorbance is measured from a reference absorbance which is constant over a considerable pH range at the lower pH values. In order to make the calculations consistent among themselves, the changes in absorption were always related to the maximum absorption of the corresponding curves. From many ultraviolet absorption

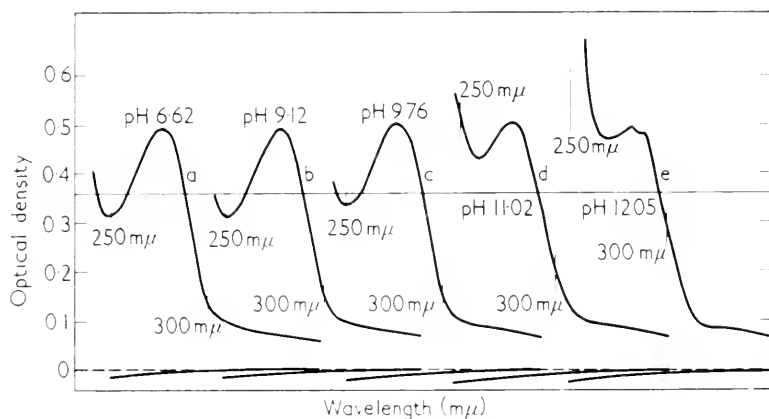


FIG. 6. The variation in the ultraviolet absorption spectrum of protochlorophyll holochrome with pH.

curves, such as those presented in Fig. 6, pK values were calculated from changes in the absorbancies at 300 mμ and at the minimum near 250 mμ prominent in the left-hand curve. The two values obtained were 11.0 and 10.4, respectively.

The changes in absorption at 300 mμ and the pK value obtained certainly implicate tyrosine as one of the amino acids undergoing ionization in the holochrome. The changes at the absorption minimum could possibly be ascribed to cysteine [14] although this is by no means certain. The pK value of 10.4 approximates to that reported for cysteine, 9.1 to 10.8 [12].

ULTRAVIOLET IRRADIATION AND PROTOCHLOROPHYLL TRANSFORMATION

A further reason for assuming that protochlorophyll is attached to more than one amino acid is the effect of exposing the holochrome to

ultraviolet radiation. Mr. G. C. McLeod and Miss J. Coomber, in our laboratory [15], discovered that protochlorophyll holochrome irradiated with various ultraviolet wavelengths between 250 and 330 $m\mu$ converted only 25 to 30% of the protochlorophyll transformed at 366, 436 $m\mu$ (Fig. 7), or with visible light from an electric lamp. If after ultraviolet irradiation, however, the holochrome solution was placed in visible light, the same degree of transformation was achieved as if no previous conversion with ultraviolet had occurred. Wherefore, the ultraviolet at the intensities used had no ill effect on the transformation.

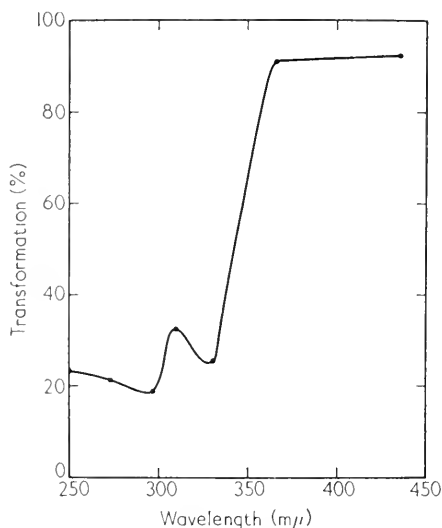


Fig. 7. The maximum conversion of holochromatic protochlorophyll to chlorophyll in the range 436 to 250 $m\mu$.

The conversion with ultraviolet light could not be explained by protochlorophyll absorption else the conversion would have been augmented with longer exposures. But the exposures given were two or three times those necessary to achieve maximum conversion in the 250–330 $m\mu$ range.

The limited action of ultraviolet light may be reasonably explained by assuming the protochlorophyll to be activated through transfer of the energy absorbed by a closely associated amino acid. Only the aromatic amino acids absorb appreciably throughout this range, and of these acids only tyrosine has the proper pK value to correspond with the alkalinities effective in the inhibition of the transformation. From these considerations it is concluded that about 25 to 30% of the protochlorophyll is attached to protein in the holochrome through the tyrosinyl group.

INFLUENCE OF pH ON THE SPECTRAL ABSORPTION OF PROTOCHLOROPHYLL IN THE HOLOCHROME

If alkalinity affects the association of protochlorophyll with protein, the absorption spectrum of protochlorophyll in the holochrome should vary with pH. This deduction comes from the fact that the absorption spectra of protochlorophyll in the free and holochromatic states differ.

The absorption spectrum of protochlorophyll does vary with pH. It shifts to shorter wavelengths with higher pH as the results of Table I show. This is what would be expected if dissociation were greater at higher alkalinities.

TABLE I
EFFECT OF pH VALUES ON THE ABSORPTION MAXIMUM
OF PROTOCHLOROPHYLL HOLOCHROME

pH	Wavelength of absorption max.
9.16	639.5
9.70	637.5
10.27	637.5
10.88	634.5
11.92	633.5

DISRUPTION OF THE CYCLOPENTANONE RING

When protochlorophyll holochrome is treated at alkalinities near pH 11, the absorption spectrum of the protochlorophyll changes drastically in the blue region of the spectrum. The absorption band at 421 m μ is increased in height at the expense of the 440 m μ band. The spectrum obtained, Fig. 8, is similar to that obtained by Granick [16] for protoporphyrin and magnesium protoporphyrin (cf. insert Fig. 8), which indicates the conversion of pheoporphyrin to porphyrin. Whether this disruption of the cyclopentanone ring takes place before or after the splitting of the pigment-protein complex is being examined at the present time.

Summary

Measurements on the fluorescence polarization of protochlorophyll holochrome have led to the supposition that protochlorophyll is attached to amino acid "tails" of the protein in the holochrome. This supposition has been strengthened by determinations on the inhibition of the transformation by alkali. The results of these determinations indicate the involvement of several amino acids in this pigment-protein binding. Comparison of the pH values effective in preventing the transformation

with the pK values of various amino acids suggests the participation of lysine, cysteine, and tyrosine. Changes in ultraviolet absorption of the holochrome with pH implicates cysteine and tyrosine. Furthermore, the limited transformation produced by ultraviolet radiation points strongly

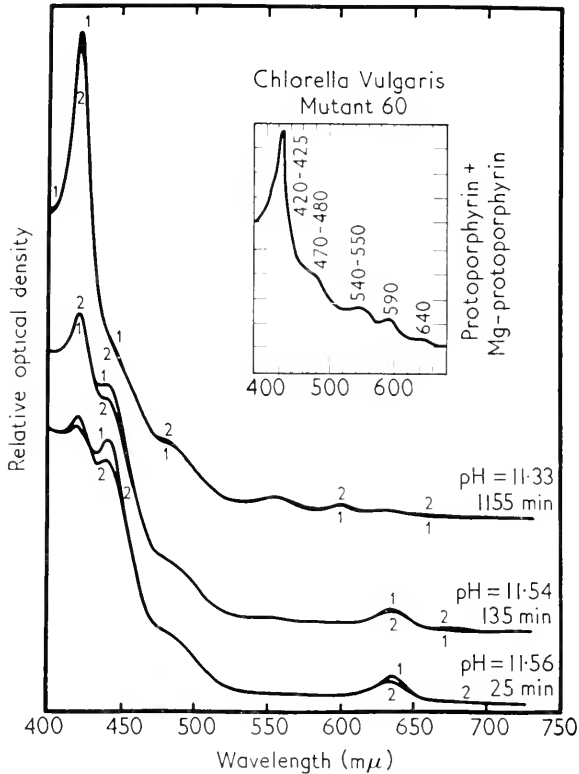


FIG. 8. The effects on the absorption spectrum and transformation of the protochlorophyll holochrome caused by extended treatment at high pH.

to tyrosine as binding from 25 to 30% of the protochlorophyll. The shift of the absorption spectrum of protochlorophyll holochrome in the visible with increased pH values also implies a disturbance of the linkage between pigment and protein. pH values of 11 and above cause rapid splitting of the cyclopentanone ring. How far this controls the inhibition of transformation is yet to be determined.

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Discussion

GOODWIN: It doesn't appear that carotenoids play any important part in the normal transformation of protochlorophyll into chlorophyll. I was wondering if they can play a part if required. In other words have you run an action spectrum for this transformation and can the carotene-absorbed light be used?

SMITH: Actually the carotene-absorbed light is a hindrance, because it acts as a screen. When you run the action spectrum you find that it is exactly the absorption spectrum of protochlorophyll in the albino leaf, and in a normal leaf which contains large amounts of carotenoids the peak in the violet is very low compared to the peak in the red. But the action spectrum in an albino plant is very high in the violet as compared to that in the red, so consequently the carotenoids actually act as a screen.

CHANCE: Since you appear to have one chlorophyll per particle do you then consider that you have a heterogeneous distribution of particles or two separate bonds on a single particle?

SMITH: We presume a distribution of particles and this is probably right because otherwise you would expect the transfer of energy, once it is absorbed, through the whole protein to carry on the transformation, but since there are discrete particles and these particles are 80 to 100 Å in diameter you can't get energy transfer very well between the particles.

KAMEN: How do you conceive the process of chlorophyll formation?

SMITH: I wish we knew the answer to that question. I pointed out that you have a change in the absorption spectrum of chlorophyll in the plant after it is formed. We are thinking that perhaps it is formed on one protein and transferred to another, but it must go onto the same protein molecule or else you would not get the increase in depolarization that you do. If it went onto separate molecules you wouldn't get this depolarization linear with concentration, but since you do get that you are piling them up on the same protein molecule, and the explanation that we have of this is that owing to the change in absorption spectrum and owing

to this polarization effect you are actually making them on one enzymic particle and moving them over to another. We have no experimental evidence on this.

FRENKEL: Are there two types of protochlorophyll on your particles, one phytylated and one non-phytylated?

SMITH: There are no phytylated compounds present. All we have is the non-phytylated.

FRENKEL: I wonder if anyone has carried out an experiment yet to ascertain whether the hydrogens in the transformation of protochlorophyll to chlorophyll *a* come from water or from some non-exchangeable hydrogens on the protein?

SMITH: We did do this a number of years ago when we had high hopes that protochlorophyll would be the photosynthetic hydrogen acceptor. We did this by the Pringsheim method of quenching of phosphorescence of tryptoflavin. We put etiolated leaves into the apparatus and pumped off all the oxygen so that we had no quenching of the phosphorescence. Then we illuminated the leaves and although the transformation of the protochlorophyll was 80%, complete, we got off the leaves only 1 or 2% of the theoretical amount of oxygen. In other words the hydrogen did not seem to be pulled away from the water. Now if you went ahead with this and then had the chlorophyll already formed and put those leaves in, then on illumination the oxygen just rolled off, so there is a time factor involved, so the failure to produce oxygen by the initial illumination can't be just the question of utilization of that oxygen by respiration.

Photosynthetic Phosphorylation and the Energy Conversion Process in Photosynthesis*

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1. Photosynthesis outside the living cell

It is fitting to recall in a symposium devoted to biological structure and function, that understanding of life phenomena at a molecular level was always advanced by the separation of a physiological process from the structural complexity of the living cell. This happened first for fermentation when Büchner in 1897 prepared from yeast a cell-free juice that fermented sugar [2]. The most recent example is the cell-free synthesis of DNA by Kornberg [3], a development which demonstrated that the key events in reproduction can be investigated with isolated enzyme systems.

With regard to photosynthesis, in 1953 Rabinowitch wrote that "the task of separating it from other life processes in the cell and analyzing it into its essential chemical reactions has proved to be more difficult than was anticipated. The photosynthetic process, like certain other groups of reactions in living cells, seems to be bound to the structure of the cell; it cannot be repeated outside that structure" [4].

There was no special reason why, at that late date in the development of biochemistry, photosynthesis could not be reconstructed outside the living cell. The simplest explanation for the repeated failures was that inappropriate experimental methods had been used for this task in different laboratories, including our own [5]. A continuing search for improved experimental methods appeared therefore worth while.

The most hopeful possibility for isolating photosynthesis from the structural complexity of the whole cell seemed to lie in chloroplasts. Few physiological processes have such an obvious relation to a distinct cellular particle as photosynthesis has to chloroplasts. In all plants which have chloroplasts, not only do these particles contain all the chlorophyll (and the accessory pigments) without which photosynthesis cannot proceed,

* This article is based on a paper presented at the Symposium on "Light and Life", at Johns Hopkins University, March 28-31, 1960 [1].

† Aided by grants from the National Institutes of Health and the Office of Naval Research.

but also, the final products of this process, starch and molecular oxygen, are formed in or at the surface of illuminated chloroplasts.

Chloroplasts were once widely believed to be the site of complete photosynthesis, that is, of oxygen evolution coupled with carbon dioxide assimilation [6, 7]. But this view was not supported by critical experimental evidence and was later abandoned when Hill found in 1937 that isolated chloroplasts produce oxygen in light but cannot assimilate CO_2 [8-12]. Investigators who followed Hill corroborated his statement that "if we break the green cell, it is possible to separate the fluid containing the chloroplast and chloroplast fragments from the tissue residue. This green juice can no longer assimilate CO_2 but in the case of many plants the insoluble green material, for a time at least, is still capable of giving oxygen in light" [8].

In 1954 we found that previous difficulties in obtaining CO_2 fixation by isolated chloroplasts were indeed methodological. By using gentler techniques of isolating chloroplasts from leaves, we prepared spinach chloroplasts that were capable not only of giving the expected Hill reaction, i.e. oxygen evolution, but also of converting CO_2 to starch and sugar at physiological temperatures and with no energy supply except visible light [13-15].

Under the new experimental conditions, CO_2 assimilation by isolated chloroplasts was strictly light-dependent and proceeded at an almost constant rate for at least an hour. There was approximate correspondence between the oxygen evolved and the CO_2 fixed, as would be expected from the well-known photosynthetic quotient in green plants, $\text{O}_2/\text{CO}_2 = 1$. The products of CO_2 assimilation were found to be the same as in photosynthesis by whole cells. The insoluble product of CO_2 fixation by chloroplasts was identified as starch. Among the soluble products the following were found: phosphate esters of fructose, glucose, ribulose, sedoheptulose, dihydroxyacetone, and glyceric acid; glycolic, malic, aspartic acids, alanine, glycine and free dihydroxyacetone and glucose [14, 15]. Using similar techniques, investigators in several different laboratories have confirmed the ability of illuminated chloroplasts to form starch and sugars from CO_2 and water [cf. 16-21].

Most of the early work on extracellular photosynthesis was done with spinach chloroplasts. But more recently, the same products of CO_2 assimilation in light were also obtained with isolated chloroplasts from several different species: sugar beet, sunflower, *Phytolacca americana* and *Tetragonia expansa* [22, 23].

There was thus finally a firm experimental basis for concluding that chloroplasts are indeed the sites of complete photosynthesis in green plants. In the light of the new evidence, chloroplasts emerged as remarkably complete and autonomous cellular structures that have become

specialized for carrying out the complete process of photosynthesis in green plants. It seemed legitimate therefore to explore the component photochemical reactions in isolated chloroplasts with the expectation that they would also be relevant to photosynthetic events in intact cells.

2. The role of light in CO₂ assimilation

From a biochemical point of view, the central problem of photosynthesis was the identification of those photochemical reactions that provide the energy required for the conversion of CO₂ to carbohydrates. As for CO₂ assimilation proper, it became evident by 1956 that the early proposals of Thimann [24], Lipmann [25], and Ruben [26] about it being a dark process, were correct. Their hypotheses that CO₂ reduction in photosynthesis is a dark reaction, a reversal of the well-known oxidative reaction of glycolysis, received experimental support mainly from the work of Calvin and his associates [27], who identified phosphoglyceric acid and other well-known products of glycolysis among the early products of photosynthesis.

A special feature of CO₂ assimilation in photosynthesis was found to be the carboxylation reaction that accounted for the appearance of phosphoglyceric acid as the first stable product of CO₂ fixation. Work in the laboratories of Calvin [28], Horecker [29], Ochoa [30], and Racker [31] established the presence in photosynthetic tissues of two special enzymes, carboxydismutase and phosphoribulokinase, which accounted for the entry of CO₂ into the metabolism of photosynthetic cells by way of a five-carbon phosphorylated sugar, ribulose diphosphate. Ribulose diphosphate on combining with CO₂ is split to give two molecules of phosphoglyceric acid.

However, even this special feature of carbon assimilation was soon found in non-photosynthetic bacteria as well. In fact, Trudinger [32] and Aubert *et al.* [33] found the entire "photosynthetic carbon cycle" in the non-photosynthetic sulphur bacterium *Thiobacillus denitrificans*. It thus became clear that CO₂ assimilation is fundamentally extraneous to the photosynthetic process. All the reactions of CO₂ assimilation in photosynthesis occur also in non-chlorophyllous cells.

The carboxylation reaction resulting in the formation of phosphoglyceric acid (PGA) requires ATP, and the reduction of PGA to the level of carbohydrate requires both ATP and reduced pyridine nucleotide. The distinction between photosynthetic and non-photosynthetic cells seems to lie, therefore, in the manner in which ATP and reduced pyridine nucleotide are formed. Photosynthetic cells form these compounds at the expense of light energy whereas non-photosynthetic cells form them at the expense of energy released by dark reactions.

Before this biochemical interpretation of photosynthesis could be accepted with confidence, it was necessary to determine whether the

process of CO_2 assimilation by isolated chloroplasts followed the same pathway as in algal cells and leaves. This was done by subdividing the chloroplasts into component parts and identifying in them, or isolating from them, the individual enzyme systems that account for the conversion of CO_2 to carbohydrate [34-36]. The results have established that in isolated chloroplasts, as in whole cells, the conversion of CO_2 to carbohydrate proceeds by the same series of dark reactions that are driven by ATP and TPNH_2 (TPNH_2 , not DPNH_2 , was the reduced pyridine

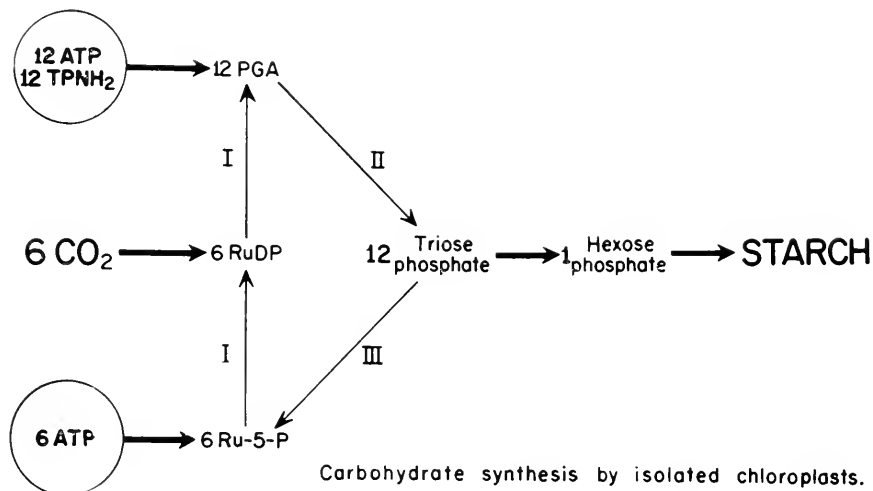


FIG. 1. Condensed diagram of the reductive carbohydrate cycle in chloroplasts. The cycle consists of three phases. In the carboxylative phase (I), ribulose-5-phosphate (Ru-5-P) is phosphorylated to ribulose diphosphate (RuDP) which then accepts a molecule of CO_2 and is cleaved to 2 molecules of phosphoglyceric acid (PGA); in the reductive phase (II) PGA is reduced to triose phosphate; in the regenerative phase (III) triose phosphate is partly converted into Ru-5-P and partly into hexose phosphate and starch. All the reactions of the cycle occur in the dark. The reactions of the carboxylative and reductive phases are driven by ATP and TPNH_2 formed in the light. One complete turn of the cycle results in the assimilation of 1 mole of CO_2 at the expense of 3 moles of ATP and 2 moles of TPNH_2 .

nucleotide formed by illuminated chloroplasts). The general scheme for CO_2 assimilation by isolated chloroplasts is summarized in Fig. 1.

The validity of the scheme shown in Fig. 1 was supported by a physical separation of the light and dark reactions of photosynthesis in chloroplasts [37]. The light phase was carried out first by the complete chloroplast system in the absence of CO_2 and resulted in an evolution of oxygen accompanied by an accumulation of TPNH_2 and ATP in the reaction mixture. The green portion of the chloroplasts (grana; cf. Fig. 2) was then discarded and when CO_2 was next supplied to the remaining non-green portion of the

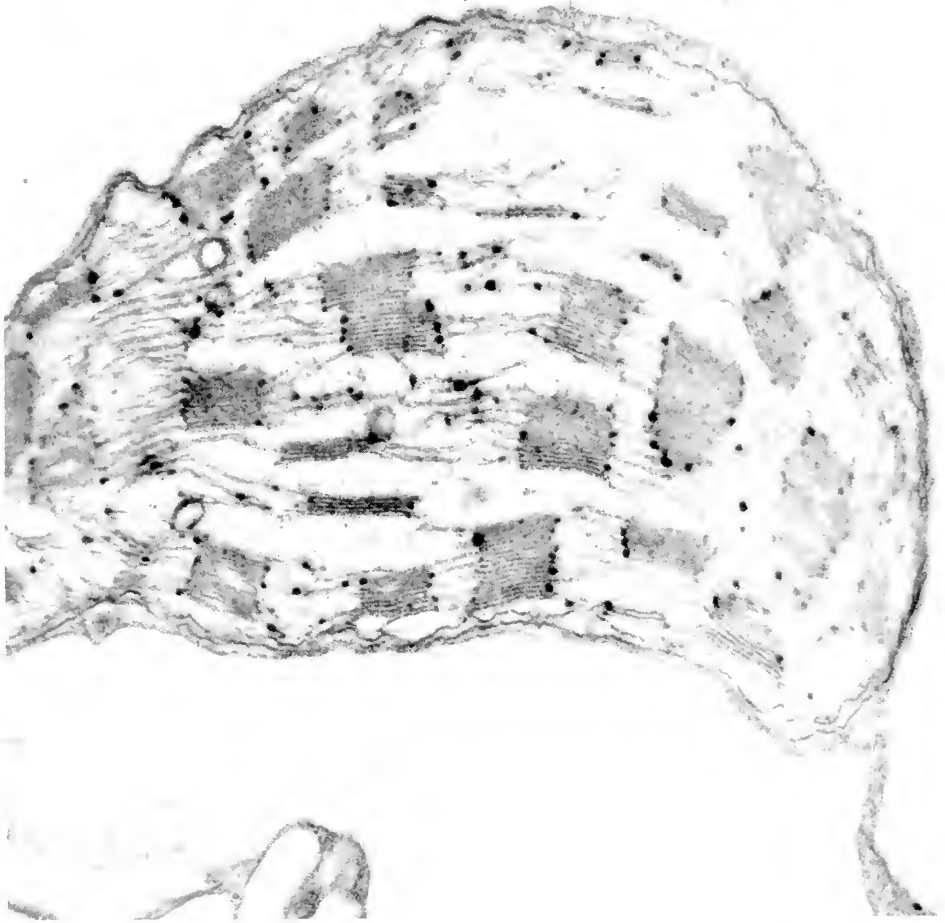


FIG. 2. Electron micrograph of a section of a maize chloroplast showing details of structure. The dense areas that resemble stacks of coins are the grana. The layers within each granum are called grana lamellae. The grana lamellae of different grana are inter-connected by stroma lamellae. Magnification 35 000 × (courtesy of Dr. A. E. Vatter).

chloroplasts [38, 39] in the *dark*, it was converted to sugar phosphates. The light and dark phases when carried out separately, yielded essentially the same final photosynthetic products as the continuously illuminated chloroplast system. The products included hexose and pentose mono- and diphosphates, phosphoglyceric acid, dihydroxyacetone phosphate, and small amounts of phosphoenolpyruvate and malate [37].

The same products of CO_2 assimilation by chlorophyll-free chloroplast extracts, including phosphorylated sugars, were also obtained in a total dark chemosynthesis where TPNH_2 and ATP were not supplied by a preceding photochemical reaction but were prepared either chemically or enzymically, or were derived from animal material [37]. (Similar experiments were carried out earlier by Racker with a multi-enzyme system that included enzymes from rabbit muscle, yeast, and spinach leaves [40, 41]).

3. Photosynthetic phosphorylation

The experiments with isolated chloroplasts have thus underlined the essence of photosynthesis in green plants, i.e. the energy conversion problem, as comprising those chloroplast reactions in which TPNH_2 and ATP are formed by light. With respect to TPNH_2 it has already been shown by several laboratories that isolated chloroplasts were capable of reducing TPN to TPNH_2 in light, with a simultaneous evolution of oxygen [42, 43, 5]. What remained to be determined was the source of ATP in photosynthesis, or more specifically, the cellular site and the mechanism by which ATP is being formed during photosynthesis. From the standpoint of cellular physiology, the important question is whether the ATP used in photosynthesis is supplied by some light-driven assimilation of inorganic phosphate that is peculiar to photosynthesis, or whether the ATP used in photosynthesis is supplied by respiration.

Before the recent investigations with isolated chloroplasts the only cytoplasmic particles known to form ATP were mitochondria, by the process of oxidative phosphorylation [44]. Oxidative phosphorylation by mitochondria has therefore usually been invoked in explaining the source of ATP used in photosynthesis (see, for example, Fig. 7 in ref. [45]; also review, ref. [46]). In early models of ATP formation in photosynthesis it was proposed that the reduction of pyridine nucleotide was carried out by illuminated chloroplasts and the resulting reduced pyridine nucleotide was re-oxidized with molecular oxygen by mitochondria [47]. This coupled chloroplast-mitochondrial system differed from conventional oxidative phosphorylation only in the source of the reduced pyridine nucleotide. In one case the pyridine nucleotide was reduced by light and in the other by a respiratory substrate. The phosphorylation reactions proper leading to the synthesis of ATP were in both cases dependent on enzymes localized in mitochondria.

This model for the generation of ATP in photosynthesis posed a serious difficulty. The most specialized photosynthetic tissue, the mesophyll of leaves, is noted for its paucity of mitochondria. Within the mesophyll cells, especially in the palisade parenchyma, chloroplasts are the dominant cytoplasmic bodies; mitochondria are few [48, 49]. It was diffi-

cult, therefore, to visualize how oxidative phosphorylation by mitochondria could generate enough ATP in leaf tissues that are noted for their vigorous photosynthetic activity.

The difficulty was removed in 1954, when isolated chloroplasts were found to synthesize ATP in light without the aid of mitochondria [13]. When conditions were so arranged that CO₂ assimilation was excluded, isolated chloroplasts used light energy for the esterification of inorganic phosphate in accord with the overall reaction:



Light-induced ATP formation in chloroplasts raised at once the question whether this process is analogous to oxidative phosphorylation by mitochondria. At least two fundamental differences were apparent. ATP formation by illuminated chloroplasts occurred without the consumption of molecular oxygen and without the addition of a chemical substrate to supply free energy needed for the formation of the pyrophosphate bonds of ATP. The term photosynthetic phosphorylation [13, 14] was therefore given to the light-induced ATP formation by chloroplasts to distinguish it from oxidative (respiratory) phosphorylation by mitochondria and the anaerobic phosphorylations at substrate level that occur in glycolysis. In both of these processes ATP formation occurs at the expense of energy liberated by the oxidation of a chemical substrate, whereas the only "substrate" which is being consumed in photosynthetic phosphorylation is light.

4. Photosynthetic phosphorylation in chloroplasts and bacteria

Although there was no net consumption (as measured by manometric pressure change) of molecular oxygen in photosynthetic phosphorylation, the process when first discovered, proceeded at a sustained rate only in the presence of oxygen [13, Fig. 2 (b)]. Oxygen seemed to act as a catalyst in photosynthetic phosphorylation, not as a substrate, as it does in oxidative phosphorylation. A decisive difference between photosynthetic and oxidative phosphorylation was the inability of chloroplasts to form ATP in the dark by oxidizing hydrogen donors of oxidative phosphorylation with molecular oxygen [50].

Further investigation of photosynthetic phosphorylation by spinach chloroplasts soon resulted in the identification of FMN and vitamin K as catalysts in the process [51, p. 6326; 52, 53]. At optimal (but still catalytic) concentrations of either FMN [53] or vitamin K (Fig. 3), photosynthetic phosphorylation became independent of external oxygen and proceeded vigorously in an atmosphere of nitrogen or argon. At a much lower, "microcatalytic", concentration of the added cofactors, photosynthetic phosphorylation remained dependent on oxygen, although still showing no net oxygen consumption.

These findings are in agreement with the recent results of Wessels [54], Jagendorf and Avron [55] and Nakamoto, Krogmann, and Vennesland [56], that photosynthetic phosphorylation with suboptimal amounts of cofactors is oxygen-dependent but becomes oxygen-independent at higher concentrations of cofactors.

In charting their subsequent investigations Arnon and his associates laid special stress on the anaerobic photosynthetic phosphorylation which proceeds in isolated chloroplasts at optimal catalytic concentrations of FMN and vitamin K. They considered this type more fundamental to photosynthesis in general than the oxygen-catalyzed type because it would also apply to bacterial photosynthesis, in which oxygen cannot be involved.

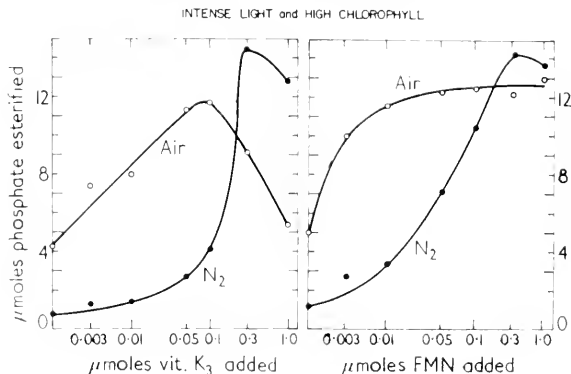


FIG. 3. Effect of vitamin K_3 (2-methyl-1,4-naphthoquinone) and FMN concentration on cyclic photophosphorylation by spinach chloroplasts in nitrogen and air at high light intensity. The reaction mixture (3 ml. final volume) included chloroplast fragments (C_{18}) containing 1.5 mg. chlorophyll; and in micromoles: tris buffer pH 8.3, 80; $K_2H^{32}PO_4$, 20; ADP, 20; $MgSO_4$, 5; and TPN, 0.3 (only in the FMN series). FMN and vitamin K_3 were added as indicated. The reaction was run for 5 min. at an illumination of 50 000 Lux (Tsujiimoto, Hall, and Arnon [92]; Arnon, Whatley, and Allen, unpublished data, 1954).

Soon after the discovery of photosynthetic phosphorylation in isolated chloroplasts, Frenkel [57] reported a similar phenomenon in the photosynthetic bacterium *Rhodospirillum rubrum*. Although Frenkel suggested that the light-induced ATP formation in bacterial preparations was similar to that in chloroplasts, the similarity seemed uncertain at first, because Frenkel's photophosphorylation system, which was a sonic macerate of *R. rubrum* cells, differed in several respects from its counterpart in isolated chloroplasts [13]. Frenkel's preparations became substrate-dependent after washing; the rate of phosphorylation was doubled on adding α -ketoglutarate [57]. But in later experiments he ruled out the dependence on an added chemical substrate [58] and the equivalence of chloroplast and bacterial photophosphorylation seemed probable.

Frenkel's findings were followed by those of Williams [59] who demonstrated photosynthetic phosphorylation in cell-free preparations of the obligately anaerobic photosynthetic bacteria, *Chromatium* and *Chlorobium*. It thus became clear that a common *anaerobic* mechanism for a light-induced phosphorylation, that does not depend on an exogenous chemical substrate or on oxygen consumption, is shared by both green plants and photosynthetic bacteria. The energy conversion process proper seemed to be fundamentally independent of oxygen although it was still possible that details of mechanisms were different in green plants and photosynthetic bacteria.

The discovery of photosynthetic phosphorylation in chloroplasts by Arnon *et al.* [13] and in bacterial particles by Frenkel [57] was confirmed and extended in a number of laboratories. Photosynthetic phosphorylation in isolated chloroplasts was observed by Avron and Jagendorf [60, 61], Wessels [62], and Vennesland and her associates [63, 56]; in algae by Thomas and Haans [64] and Petrack [65]; and in photosynthetic bacteria by Geller [66], Kamen, and Newton [67] and Anderson and Fuller [68]. In later experiments Whatley *et al.* [22, 23] have shown that photosynthetic phosphorylation by chloroplasts, which had previously been almost entirely limited to observations on chloroplasts isolated from one species, viz. spinach, is also operating in chloroplasts isolated from several other species of higher plants.* It now seems well established, therefore, that all photosynthetic organisms contain a phosphorylating system that is intimately associated with, and structurally bound to, the chlorophyll pigments.

Soon after the demonstration of photosynthetic phosphorylation in isolated chloroplasts attempts were made to compare its rate with that of CO₂ assimilation by illuminated whole cells. Since, as with most newly isolated processes in cell-free systems,† the rates of photosynthetic phosphorylation were rather low, there was little inclination at first to accord this process quantitative importance [72, pp. 292, 345] as a mechanism for converting light into chemical energy.

With further improvement in experimental methods we obtained rates of photosynthetic phosphorylation up to 170 times higher [73] than those

* Other accounts of the discovery of CO₂ assimilation and photosynthetic phosphorylation by isolated chloroplasts are given by Calvin. In 1956 he ascribed (69, p. 31) the discovery of CO₂ assimilation by isolated chloroplasts to Boychenko and Baranov (70) and in 1959 he ascribed the discovery of both CO₂ assimilation by isolated chloroplasts and photosynthetic phosphorylation to his own laboratory (71, p. 152).

† The most recent instance of this kind is the cell-free synthesis of DNA investigated by Kornberg. "The first positive results represented the conversion of only a very small fraction of the acid-soluble substrate into an acid-insoluble fraction (50 or so counts out of a million added)" [3].

originally described [13] and even these high rates were exceeded by Jagendorf and Avron [74]. The improved rates of photosynthetic phosphorylation were equal or greater than the maximum known rates of carbon assimilation in intact leaves. It appeared, therefore, that the enzymic apparatus for photosynthetic phosphorylation that is present in chloroplasts, can under appropriate experimental conditions, function outside the organized cell without substantial loss of activity.

Unlike the phosphorylating system, the enzymes catalyzing CO_2 assimilation are water-soluble [38, 39, 37] and are therefore partly lost during the isolation of chloroplasts. This results in lower rates of CO_2 assimilation by isolated chloroplasts than by the intact parent leaves. The difference between the rate of CO_2 assimilation by isolated chloroplasts and that of intact leaves may be made to appear greater, though less relevant, if the comparison is made not between isolated chloroplasts and their parent leaf tissue, but between isolated chloroplasts and unrelated leaf material that gave maximum rates of CO_2 assimilation under different experimental conditions. Nevertheless, the now known rates of CO_2 assimilation in isolated chloroplasts (10 to 20% of that in parent leaf tissue [20, 35]) are substantial enough to strengthen the conclusion that photosynthesis by isolated chloroplasts mirrors that in the intact leaf. This conclusion is fortified by the identity of the photosynthetic products found in both cases.

5. Catalysts of photosynthetic phosphorylation

Photosynthetic phosphorylation emerged as a major mechanism for converting light into useful chemical energy independently of CO_2 assimilation. It became important therefore to investigate systematically the mechanism of this direct conversion of light into pyrophosphate bond energy. The first question that received attention was the identity of the catalysts.

In searching for catalysts of photosynthetic phosphorylation by isolated chloroplasts special attention was given to normal constituents of chloroplasts and green leaves. The first factors which were found to stimulate cyclic photophosphorylation without themselves being consumed in the reaction were magnesium ions and ascorbate [13, 51]; the next to be recognized were, as already mentioned, FMN and vitamin K compounds [53, 52]. Magnesium and ascorbate have long been known to be present in chloroplasts [75]. FMN is widely distributed in green leaves [76]. Ohta and Losada in our laboratory (unpublished data) have found FMN to be a regular constituent of chloroplasts. Of unusual interest, however, was the antihæmorrhagic factor, vitamin K, which occupied, since its discovery in plants, a unique position among other vitamins in

being specifically associated with chloroplasts* [77]. Moreover, Martius and others have recently assigned a role to vitamin K in oxidative phosphorylation [82, 83; cf. 84, review; 85].

Apart from the catalytic effect of FMN and vitamin K (and TPN; cf. [86, 87]), photosynthetic phosphorylation may also be increased by the addition of non-physiological cofactors [74; cf. [88, 89]. Among these of particular interest is phenazine methosulphate, since this dye is known to be a strong reducing agent for cytochromes [90]. Phenazine methosulphate was found to stimulate photosynthetic phosphorylation in bacterial preparations by Geller [66] and Kamen and Newton [67] and in spinach chloroplasts by Jagendorf and Avron [74].

COFACTORS OF BACTERIAL PHOTOPHOSPHORYLATION

Of the cofactors of photosynthetic phosphorylation discussed so far, ascorbate and phenazine methosulphate were found to be effective in photosynthetic phosphorylation by cell-free preparations from *Rhodospirillum rubrum* [66] and *Chromatium* [67]. In addition, Geller [66] has also found a stimulatory effect of vitamin K₃.

Under our experimental conditions photosynthetic phosphorylation by cell-free preparations of *Chromatium* showed no response to added cofactors when the particles were freshly prepared under anaerobic conditions. On ageing, however, an effect of added vitamin K and phenazine methosulphate was observed (Table I); the joint addition of these two cofactors gave a greater increase of phosphorylation than when they were added singly. The addition of FMN gave no increase [66, 67] and in fact, under our experimental conditions often inhibited photosynthetic phosphorylation by *Chromatium* particles.

Table II shows that photosynthetic phosphorylation by *Chromatium* particles also resembled that of chloroplasts in its resistance to inhibition by dinitrophenol, *o*-phenanthroline and antimycin A (when phenazine methosulphate was present in the reaction mixture [cf. 66]) and its sensitivity

* Bishop, who earlier presented evidence that vitamin K is an essential factor for the photochemical activity of isolated chloroplasts [78], has reported in a more recent publication [79] that spinach chloroplasts do not contain naphthoquinones of the vitamin K type but contain instead the benzoquinone Q-255 ("plastoquinone"), which Crane [80] and Folkers and his associates [81] found in green tissues and which Crane also found to be specifically concentrated in chloroplasts [80]. Bishop [79] has reported that Q-255 activates the Hill reaction. The role of Q-255 in photosynthetic phosphorylation is still unknown, but it should be noted that from the standpoint of the mechanism of photosynthetic phosphorylation (see next Section), either a naphthoquinone of the vitamin K type or a benzoquinone would appear suitable as an electron carrier in the process. A clarification of the disagreement between the earlier reports of vitamin K distribution in chloroplast [77] and the recent reports on Q-255 will be awaited.

TABLE I

EFFECT OF VITAMIN K_3 AND PHENAZINE METHOSULPHATE (PMS)
ON PHOTOPHOSPHORYLATION BY AGED CELL-FREE PREPARATIONS OF *Chromatium*
[CHROMATOPHORES (P) AND SUPERNATANT FLUID (S)]

(Ogata, Nozaki, and Arnon [91])

Treatment*	μ moles P esterified/mg. chlorophyll/hr.				
	Ageing time (days)				
	0	1	2	4	8
1. P + S	83	55	42	40	26
2. P + S, vit. K_3 , PMS	99	98	91	80	66
3. PS	87	30	27	21	15
4. PS, vit. K_3 , PMS	96	106	75	65	45

* P and S were stored separately in Treatments 1 and 2 and together in Treatments 3 and 4.

Each vessel contained, in a final volume of 3.0 ml., cell-free preparation containing 0.3 mg. bacteriochlorophyll, and the following in micromoles: tris buffer, pH 7.8, 80; $MgCl_2$, 5; $K_2H^{32}PO_4$, 10; and ADP, 10. 0.1 μ mole each of vitamin K_3 (2-methyl-4-amino-1-naphthol hydrochloride) and PMS were added as indicated. Gas phase was argon. The reaction was carried out at 20° for 30 min. and stopped by adding 0.3 ml. of 20% TCA to each vessel. Illumination 35 000 Lux.

TABLE II

EFFECT OF INHIBITORS ON PHOTOPHOSPHORYLATION BY *Chromatium* PARTICLES

(Ogata, Nozaki, and Arnon [91])

Treatment	μ moles P esterified/ mg. chlorophyll/hr.
Control	75
10^{-3} M dinitrophenol	52
5×10^{-5} M <i>o</i> -phenanthroline	50
Control	126
Antimycin A, 10 μ g.	119
Gramicidin, 40 μ g.	119
10^{-4} M methylene blue	100
10^{-3} M <i>p</i> -chloromercuribenzoate	14

Each vessel contained, in a final volume of 3.0 ml., cell-free preparation (PS) containing 0.2 mg. bacteriochlorophyll and the following, in μ moles: tris buffer, pH 7.8, 80; $MgCl_2$, 5; $K_2H^{32}PO_4$, 15; ADP, 15; vitamin K_3 (2-methyl-1,4-naphthoquinone), 0.3; and phenazine methosulphate, 0.1. Inhibitors were added as indicated. Other conditions were the same as described for Table I.

to *p*-chloromercuribenzoate [cf. 89]. However, unlike chloroplasts [50, 88, 89], photophosphorylation by *Chromatium* particles was, under our experimental conditions, resistant to inhibition by methylene blue [cf. 66].

6. The electron flow mechanism of photosynthetic phosphorylation

Photosynthetic phosphorylation has provided direct experimental evidence for the view that the key event in photosynthesis, the conversion of light into chemical energy, is independent of the classical manifestations of this process in green plants; oxygen evolution and CO₂ assimilation. If it is accepted that photosynthetic phosphorylation represents the simplest common denominator of photosynthesis in green plants and bacteria, then a mechanism for this process would be expected to provide a basic pattern for the conversion of light into chemical energy. The salient facts which must be explained are that a "high energy" pyrophosphate bond is formed at the expense of absorbed light energy. There is no need, *a priori*, to connect this reaction either with photolysis of water or with reduction of CO₂. Photosynthetic phosphorylation catalyzed, for example, by phenazine methosulphate or vitamin K, produces neither a reductant for CO₂ assimilation nor molecular oxygen; the sole product is ATP.

The simplest hypothesis to account for the formation of ATP in photosynthetic phosphorylation is to assume that, as in the dark phosphorylations of glycolysis and respiration, the formation of a pyrophosphate bond is also coupled with a release of free energy which occurs during electron transport, i.e. when an electron drops from the higher energy level (that it has when it resides in the electron donor molecule) to the lower energy level that it assumes on joining the electron acceptor molecule. But a mechanism for photosynthetic phosphorylation must also account for its unique features: ATP is formed without the consumption of an exogenous electron donor and electron acceptor. Unlike oxidative phosphorylation, photosynthetic phosphorylation consumes neither exogenous substrate nor molecular oxygen, only light energy.

A mechanism for photosynthetic phosphorylation must, therefore, provide for the generation of both an electron donor and an electron acceptor in the primary photochemical act when radiant energy is absorbed by chlorophyll. Investigations of photosynthesis at the cellular level, in which the main preoccupation has usually been with CO₂ assimilation and oxygen evolution, led to no cogent theory of the primary act of photosynthesis that would fit the experimental facts of photosynthetic phosphorylation. As summed up recently by Livingston "physiologists and biochemists appear to believe that this question (the primary act of

photosynthesis) was answered long ago by physicists while physicists find the problem distressingly complicated and therefore uninteresting" [93].

The mechanism of photosynthetic phosphorylation that we have proposed [94] regards the photosynthetic particle, chloroplast or bacterial chromatophore, as a "closed" catalytic system. We have suggested that during the primary photochemical act, one component of the "closed" system, chlorophyll (bound to protein), becomes excited on absorbing a photon and "expels" one of its electrons that has been raised to a higher energy level. The excited chlorophyll thus becomes the electron donor. On losing an electron, chlorophyll assumes a positive charge, and in this way also becomes the electron acceptor in photosynthetic phosphorylation.

The "expelled" electron returns in a stepwise manner to the oxidized chlorophyll molecule which thereupon resumes its normal ground state. On its return "downhill" path, the expelled electron releases free energy as it passes through several electron carriers. These intermediate electron carriers are coupled with enzyme systems that catalyze the phosphorylation process during which electron energy is converted into pyrophosphate bond energy. After returning to chlorophyll, the cyclic journey of the electrons begins once more as chlorophyll molecules acquire fresh excitation energy by recurrent absorption of photons. The stepwise interaction of the "activated" electron with the intermediate electron acceptors constitutes the energy conversion process in photosynthetic phosphorylation. Because of the cyclic path travelled by electrons that are activated by light, this type of photosynthetic phosphorylation has been called *cyclic photophosphorylation* [95, 94].

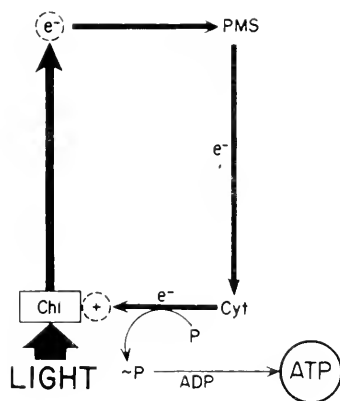
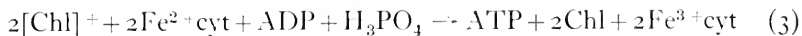
Chlorophyll can, of course, also be restored to the ground state without the excited electron going through the enzymic "energy transformer stations", but in that case electron energy has not been converted into chemical energy and hence photosynthesis has not occurred. Instead, the energy of electronic excitation is emitted as a light quantum and the characteristic fluorescence of chlorophyll is observed.

The primary photochemical reaction in which an absorbed light quantum "excites" a chlorophyll molecule and "expels" an electron, is represented by equations (2) and (2a). The symbol $[\text{Chl}]^+$ is intended to denote that the chlorophyll molecule as it loses an electron, acquires a positive charge, i.e. becomes "oxidized" or forms a "hole" ("odd ion", [96]) that is ready to accept another electron and to return in this way to its normal ground state.



In the proposed mechanism of cyclic photophosphorylation $[\text{Chl}]^+$ is restored to its ground state by accepting an electron from a cytochrome

present in the photosynthetic particle [94]. This "terminal" cytochrome component, i.e. a cytochrome that is adjacent to, and interacts with, the excited chlorophyll molecule, becomes oxidized after donating an electron to chlorophyll. We have visualized [94] that phosphorylation is coupled with the oxidation of the terminal cytochrome, in a manner analogous to the phosphorylations which accompany the oxidation of cytochromes by oxygen in mitochondria [44]. Thus, chlorophyll (with the aid of light) is the ultimate oxidant in photosynthetic phosphorylation and plays a part which corresponds to that of molecular oxygen in oxidative phosphorylation [cf. 97]. The terminal phosphorylation reaction is represented by equation (3).



Cyclic photophosphorylation (PMS type)

FIG. 4. Scheme for anaerobic cyclic photophosphorylation catalyzed by phenazine methosulphate (PMS). Details in the text.

The photophosphorylation reaction would leave the cytochrome in the oxidized state. Since cytochromes are present in catalytic amounts, cyclic photophosphorylation would soon cease unless the cytochrome could become reduced again. Our theory provides that in cyclic photophosphorylation the reduction of cytochrome occurs by the return of the electron originally "expelled" from chlorophyll in the primary photochemical reaction (equations (2) and (2a)).

In isolated chloroplasts, the reduction of cytochrome by the electron expelled from chlorophyll requires an added catalyst, i.e. an intermediary electron carrier. In the simplest case, as shown in the scheme in Fig. 4, the part of the electron carrier is played by a non-physiological catalyst, phenazine methosulphate. Phenazine methosulphate is known to be a very

effective electron carrier in reactions involving cytochromes. For example, Massey [90] has found that cytochrome *c* is rapidly reduced in a non-enzymic reaction with reduced phenazine methosulphate. In Fig. 5, the intermediary electron carrier is vitamin K or FMN.

The cyclic electron flow diagrams, illustrated by Figs. 4 and 5, are components of the scheme presented earlier [94]. The key reaction in the proposed mechanism, the photo-oxidation of chlorophyll by the loss of an electron, is based on a type of reaction in photochemistry that was experimentally documented by Lewis and Lipkin [96]. They found, by illuminating a variety of substances in rigid media, "that one of the commonest photochemical processes is the mere loss of an electron by an activated molecule" [96]. The evidence for the then (in 1942) "new and

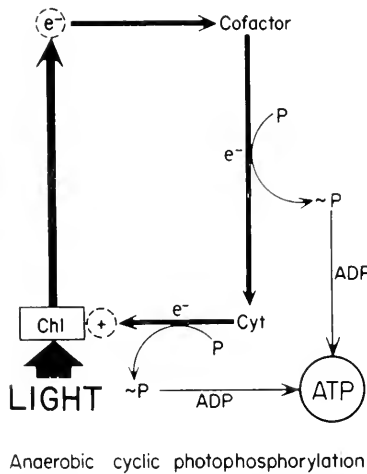


FIG. 5. Scheme for anaerobic cyclic photophosphorylation catalyzed by vitamin K_3 or FMN. Details in the text.

somewhat surprising phenomenon" [96] was, for example, "that chemical oxidation at room temperature and photo-oxidation at liquid air temperature (of tri-*p*-tolylamine) have given the same substance, namely, the positive ion left, (*p*- $\text{CH}_3\text{C}_6\text{H}_4$) $_3\text{N}^+$, when one electron has been removed" [96].

In the reactions studied by Lewis and Lipkin the fate of the ejected electron was uncertain but, as they pointed out, "the electron must lie in a potential hole which is deep enough so that the large electrostatic field of the ion is unable to dislodge it" for considerable periods of time. This was indicated by the fact that "the (blue) color (of the 'odd ion' formed) persists at liquid air temperature for several days, but at only slightly higher temperatures the color disappears. Then presumably the electron

has returned to the ion" [96]. In cyclic photophosphorylation the electron expelled from chlorophyll is visualized as being transferred [98] to the first intermediary acceptor in the photosynthetic electron transport chain and thus initiating the electron transfer process that makes cyclic photophosphorylation possible [94].

To summarize, then, the simplest experimentally demonstrable case of conversion of light energy into chemical energy, a case that is common to all chlorophyll-containing particles, is cyclic photophosphorylation. We visualize that in cyclic photophosphorylation electrons flow from chlorophyll that becomes excited by light, to a cofactor (Figs. 4 and 5), from the cofactor to cytochromes and from cytochromes back to chlorophyll. During this cyclic flow of electrons the cofactor and cytochromes present in the photosynthetic particles undergo oxidation-reductions which are believed to be coupled to phosphorylation reactions that produce ATP.

The proposed mechanism for this process may be divided into three phases: (*a*) the primary photochemical act that results in the generation by the excited chlorophyll molecule of a high energy electron and of the ultimate electron acceptor [Chl⁺], (*b*) transport of the high energy electron through a photosynthetic electron transport system, and (*c*) phosphorylation reactions coupled to electron transport. Phases (*b*) and (*c*) are analogous and possibly identical in some respects with their counterparts in oxidative phosphorylation, whereas phase (*a*) is peculiar to photosynthetic phosphorylation.

7. Evidence for electron flow mechanism in cyclic photophosphorylation

The validity of the proposed mechanisms for cyclic photophosphorylation is supported by several lines of evidence. These include recent experiments on the effect of chloride and ferricyanide on photosynthetic phosphorylation in isolated chloroplasts and chromatophores, and experiments on the effect of light and vitamin K on cytochromes of chlorophyllous particles. This evidence will now be discussed in more detail.

EFFECT OF CHLORIDE

The role of chloride in photosynthesis was discovered by Warburg [99], who found that chloride, replaceable by bromide but not by other anions, was essential for oxygen evolution by isolated chloroplasts. This discovery was fully confirmed by Arnon and Whatley [100], but they were disinclined to accept, at that time, Warburg's conclusion that chloride is a coenzyme of photosynthesis, because this conclusion would have conferred on chloride the then unwarranted status of an essential element for green

plants. However, they envisaged the possibility, which has since been documented by Broyer and associates [101] and Martin and Lavollay [102], that chloride may prove to be an essential micronutrient for green plants,

A reinvestigation by Boyé *et al.* [103] of the role of chloride in the photochemical reactions of chloroplasts confirmed Warburg's conclusion that chloride is essential for those photosynthetic reactions in which oxygen is liberated. Chloride was not required, however, for the anaerobic cyclic photophosphorylation that is shared by bacterial particles and chloroplasts. Thus, in the absence of chloride, chloroplasts behaved like bacterial chromatophores. They were able to carry out the anaerobic cyclic photophosphorylation but were unable to evolve oxygen. Oxygen evolution, not included in the mechanism of cyclic photophosphorylation, appeared therefore as an additional secondary feature of photosynthesis, not essential to the primary conversion of light energy into the pyrophosphate bonds of ATP, and peculiar to green plants.

EFFECT OF FERRICYANIDE

The key premise in the proposed mechanisms for cyclic photophosphorylation is that the electron expelled from the chlorophyll molecule in the primary photochemical act is not removed from the "closed circuit" within which it travels before it returns to the chlorophyll. If this basic postulation is correct it follows that cyclic photophosphorylation should be abolished if the electrons are prevented from completing the cycle because of capture by an external electron acceptor. To be convincing, such an experiment should be carried out with an electron acceptor which would be free from the suspicion that it prevented phosphorylation by acting as an uncoupler, or in some toxic manner.

An electron acceptor that fulfills these requirements is ferricyanide. As shown by Jagendorf [104], and confirmed in this laboratory, ferricyanide has a great affinity for trapping electrons during photophosphorylation. Thus, by adding ferricyanide, in the absence of chloride, cyclic photophosphorylation in both chloroplasts and chromatophores should be inhibited, if the proposed hypothesis is correct. The cyclic flow of electrons in the closed circuit would be interrupted when the electrons are trapped by, and used in, the reduction of ferricyanide.

Table III shows that this theoretical prediction has been experimentally verified. The addition of ferricyanide abolished cyclic photophosphorylation both in chloroplasts and in chromatophores. Adding this ion in its reduced form as ferrocyanide, was without effect. The reduction of ferricyanide with ascorbate either prior to, or during illumination of the photosynthetic particles, restored in full their capacity for cyclic photophosphorylation. The conclusion seemed justified therefore that the

TABLE III

INFLUENCE OF FERRICYANIDE (IN THE ABSENCE OF CHLORIDE) ON CYCLIC PHOTOPHOSPHORYLATION BY SPINACH CHLOROPLASTS AND BACTERIAL CHROMATOPHORES (*Chromatium*) (μ MOLES PHOSPHATE ESTERIFIED IN 30 MIN.)

(Bové, Bové, Whatley, and Arnon [103])

Treatment	Chloroplasts	Chromatophores
Control	9.2	4.9
Ferricyanide, 1 μ mole	0.5	0.4
Ferricyanide, 2 μ moles	0.5	0.5
Ferricyanide, 3 μ moles	0.5	0.4
Ferricyanide, 5 μ moles, reduced by ascorbate*	7.2	6.2
Ferrocyanide, 5 μ moles	9.4	5.4

* Sodium ascorbate (5 μ moles) was tipped in from a sidearm 15 min. after the beginning of the experiment, and illumination (35 000 Lux) was then continued for 30 min.

inhibitory effect of ferricyanide resulted from the capture by this ion (in its oxidized form) of electrons which would have normally travelled the cyclic electron transport route (Fig. 5). This conclusion was strengthened by the finding that the inhibition was produced by very low concentrations of ferricyanide. This would be expected if, as demanded by the hypothesis, the quantity of ferricyanide needed to capture electrons from the cyclic system needs only to be sufficient to leave the catalytic components of the system in an oxidized form.

LIGHT-INDUCED OXIDATIONS OF CYTOCHROMES

Our theory assigns to cytochromes the role of electron carriers in photosynthetic phosphorylation. The initial suggestion [13] that cytochromes oxidized by light may act as electron carriers in the electron transport chain of photosynthetic phosphorylation was based on the observation of Lundegårdh [105] that the cytochrome peculiar to chloroplasts, cytochrome *f* [97], is oxidized on illumination. The oxidation of cytochromes on illumination has also been observed in intact algae and photosynthetic bacteria by Duysens [106, 107] and by Olson and Chance [108]. Of special relevance is the recent finding of Chance and Nishimura [109] that, in whole *Chromatium* cells, a light-induced oxidation of cytochrome *c*₂ is independent of temperature. This accords with the main postulate of our theory [94] that the primary photochemical act in photosynthesis consists of electronic excitation and is thus independent of a thermal reaction.

In illuminated cell-free preparations of *R. rubrum*, Smith and Ramirez [110] and Smith and Baltscheffsky [111] have observed changes in the absorption spectrum of cytochrome c_2 that were associated with phosphorylation. Their conclusions [111], that in the facultative anaerobe *R. rubrum* the "photosynthetic" cytochrome c_2 is not a part of the respiratory chain, and that "two different enzyme systems mediate the oxidation of substrates by oxygen and the phosphorylation of ADP by illumination",

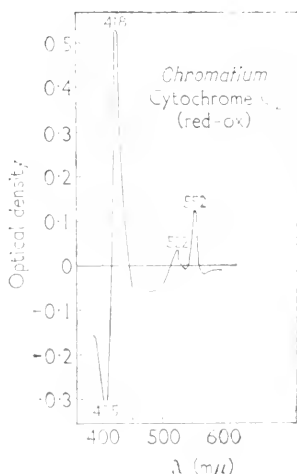


FIG. 6. Difference spectrum (reduced minus oxidized) of purified cytochrome c_2 of *Chromatium* (Nozaki, Ogata, and Arnon [114]).

are concordant with our distinction between photosynthetic and oxidative phosphorylation in green cells [14, 46, 112, 50].

A reversible light-induced oxidation of cytochrome c_2 in cell-free preparations of *Chromatium*—a cytochrome that has been isolated and purified by Bartsch and Kamen [113]—was measured by Nozaki *et al.* [114]. The absorption spectrum of the reduced form of a purified cytochrome c_2 from our preparations (Fig. 6) is the same as that described by Bartsch and Kamen [113].

The effect of light on the absorption spectrum (difference spectrum, light minus dark) of cytochromes in cell-free preparations of *Chromatium* is shown in Fig. 7. On illumination, the absorption spectrum of cytochromes shows oxidation followed by a reduction in the dark and re-oxidation on repeated illumination. Under the experimental conditions in which the *Chromatium* cell-free system was investigated, the light-dark reversible oxidation-reduction reactions were sufficiently decelerated to be conveniently measurable with a recording spectrophotometer, at room temperature.

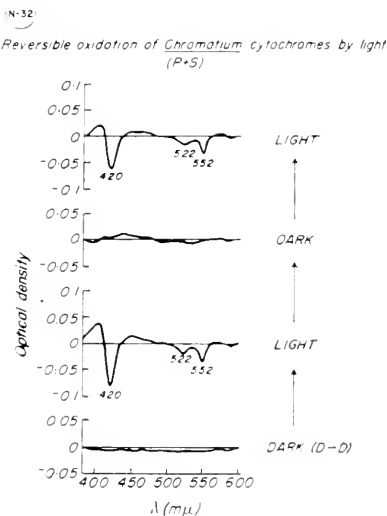


FIG. 7. Successive oxidation by light and reduction in the dark of cytochromes in cell-free preparations of *Chromatium*. The reaction mixture included in a final volume of 3.0 ml., chromatophores (P) containing 0.06 mg. bacteriochlorophyll and supernatant fluid (S) corresponding to 0.5 mg. bacteriochlorophyll. A small amount of $\text{Na}_2\text{S}_2\text{O}_4$ was previously added to S, which was then dialyzed against 0.2 M tris buffer, pH 7.8 prior to use. The reaction was carried out at room temperature. Gas phase argon. Difference spectra, using the dark treatment as control, were made in Thunberg type cuvettes, with a Cary recording spectrophotometer. Illumination was by a tungsten lamp (35 000 Lux). (Nozaki, Ogata, and Arnon [114]).

EFFECT OF VITAMIN K

In fresh preparations of chromatophores the reduction of oxidized cytochrome in the dark was not influenced by the additions of added cofactors (compare Table I). However, as shown in Fig. 8, in aged preparations, the reduction of the oxidized cytochromes was greatly accelerated by the addition of vitamin K, either in the oxidized form (vitamin K_3) or in the reduced form (vitamin K_5). The effect of vitamin K, as an electron carrier in accelerating the reduction of oxidized cytochrome depended on the presence of chromatophores. Without chromatophores, using a purified cytochrome c_2 , a hundred-fold greater concentration of reduced vitamin K was required to reduce the oxidized cytochrome.

The observed effects of vitamin K in catalyzing the reduction of cytochromes which had been oxidized in light, support the electron flow theory [94] for cyclic photophosphorylation. This theory assigns to vitamin K, or some analogous quinone, a role of an intermediate electron carrier in the electron transport chain associated with photophosphorylation.

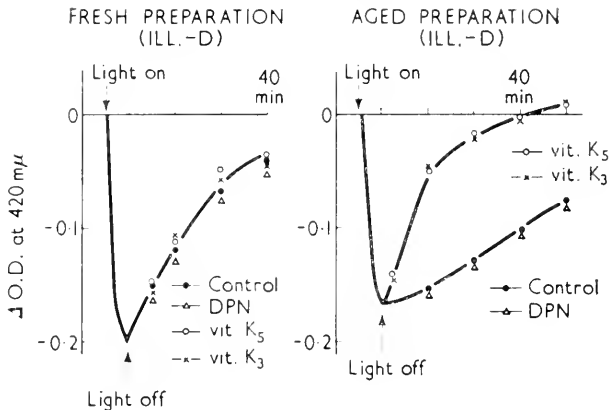


FIG. 8. Effect of vitamin K and other cofactors on the reduction of cytochrome c_2 in cell-free preparations of *Chromatium*. The cytochromes were oxidized by previous illumination (cf. Fig. 7). The reaction mixture included in a final volume of 3.0 ml., dialyzed cell-free suspension (PS) containing 0.06 mg. bacteriochlorophyll, 0.02 μ mole of purified cytochrome c_2 and 0.03 μ mole of the respective cofactors. Difference in optical density was measured on a Beckman DU spectrophotometer with an attached photomultiplier using cuvettes with the respective cofactor omitted as controls in each case (Nozaki, Ogata, and Arnon, [114]).

LIGHT-INDUCED CHANGES IN CHLOROPHYLL

New experimental evidence for the electron flow theory has come from the recent work of Arnold and Clayton [115] who, on illuminating bacterial chromatophores, observed temperature-independent (1°K to 300°K) reversible spectral changes in the absorption bands of bacteriochlorophyll. These spectral changes in chlorophyll that are independent of temperature are consistent with the proposed electron shift that would result from the primary photochemical act (compare equations (2) and (2a)).

8. Multiple sites in cyclic photophosphorylation

As already discussed, cyclic photophosphorylation is catalyzed by vitamin K and FMN and also by non-physiological factors such as phenazine methosulphate. This latter fact has given rise to questions whether vitamin K and FMN, or equivalent quinone and flavin constituents of photosynthetic tissues, are to be considered the physiological catalysts of cyclic photophosphorylation or whether they are to be regarded as non-specific agents in no way distinguishable from non-physiological catalysts. The question was of special interest in connection with phenazine methosulphate because this dye has given rates of photosynthetic phosphorylation higher than either vitamin K or FMN [cf. 74].

The marked effectiveness of phenazine methosulphate could be explained by its acting as an electron carrier that bypasses a rate-limiting step in photosynthetic phosphorylation [66]. This is suggested by the observation of Geller [66] that the severe inhibition of photophosphorylation in *R. rubrum* by antimycin A does not occur in the presence of phenazine methosulphate. On this hypothesis phenazine methosulphate might give higher rates of phosphorylation at high light intensity when there is a rapid flux of electrons from excited chlorophyll. Assuming, however, several sites of phosphorylation in the cyclic pathway, the advantage of phenazine methosulphate might disappear at *low light intensity* when the overall rate

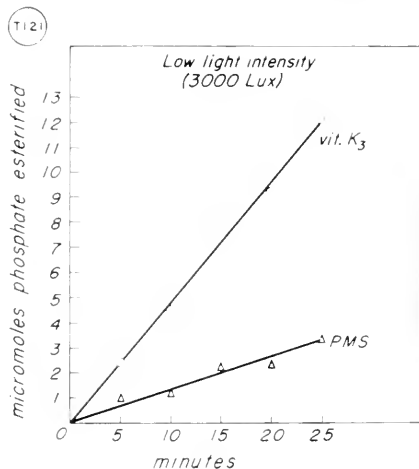


FIG. 9. Effect of vitamin K₃ and phenazine methosulphate (PMS) on anaerobic cyclic photophosphorylation in spinach chloroplasts at a limiting light intensity (3000 Lux). Reaction mixture included chloroplast fragments (C₁₂) containing 1 mg. chlorophyll and in micromoles: tris buffer, pH 8.3, 80; K₂H³²PO₄, 15; MgSO₄, 5; ADP, 15; vitamin K₃ or PMS, 0.3; gas phase nitrogen (Tsujiimoto, Hall, and Arnon [92]).

of the process is limited by the electron flux. Under such conditions the highest rate of photophosphorylation would be observed in a system in which none of the phosphorylation sites was bypassed. Thus, a comparison of photosynthetic phosphorylation, catalyzed by vitamin K and phenazine methosulphate under conditions of limiting light, seemed desirable.

The results of such a comparison are shown in Fig. 9. At low light intensity photophosphorylation catalyzed by vitamin K₃ was markedly greater than that catalyzed by phenazine methosulphate (or pyocyanin). This difference was persistent and gave a straight-line relationship for a considerable period of time.

These experiments were extended by comparing the rates of photosynthetic phosphorylation at different light intensities. The results shown in Fig. 10 confirm and extend those illustrated in Fig. 9. At low intensity cyclic photophosphorylation catalyzed by either vitamin K₃ or FMN proceeded at a much higher rate than that catalyzed by phenazine methosulphate. However, at higher light intensities the phenazine methosulphate system gave much greater rates of phosphorylation [92].

These results suggest that at high light intensity the vitamin K and FMN systems became limited by enzymic reactions which were unable to

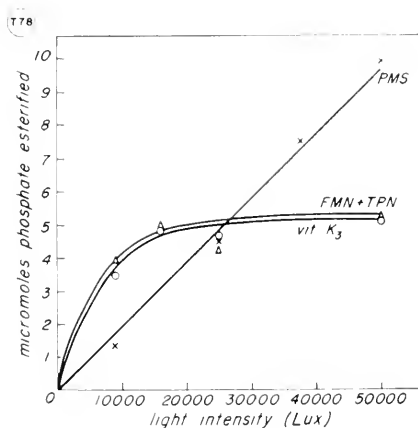


FIG. 10. Effect of light intensity on anaerobic cyclic photophosphorylation. Gas phase nitrogen. Illumination period 30 min. The reaction mixture included chloroplast fragments (C_{15}) containing 0.1 mg. chlorophyll and chloroplast extract equivalent to 1 mg. chlorophyll. 0.3 μ mole TPN and 0.3 μ mole FMN were included in the FMN system. Other conditions as given for Fig. 9 (Tsuchimoto, Hall, and Arnon [92]).

keep pace with the rapid electron flux. The increasing rates of phosphorylation obtained at high light intensity with the phenazine methosulphate system are consistent with the explanation that this agent does indeed serve as a bypass around some rate-limiting step, probably by catalyzing the reduction of cytochromes [90]. These findings are interpreted as an indication of enzymic steps that may limit cyclic photophosphorylation at high light intensities, when physiological catalysts such as vitamin K or FMN (or their analogues) are involved.

The findings that, when light is limiting, vitamin K and FMN catalyze higher rates of photophosphorylation than phenazine methosulphate (Fig. 10), suggest the involvement of at least two phosphorylation sites in the vitamin K and FMN pathways. A diagrammatic representation of this mechanism is given in Fig. 5.

A primary phosphorylation reaction, common to all pathways of cyclic photophosphorylation, is envisaged as being coupled with the oxidation of the terminal cytochrome in the photosynthetic particle, i.e. the cytochrome that reacts with the excited chlorophyll molecule (compare Figs. 4 and 5). A second site of phosphorylation in the vitamin K pathway is likely to occur on oxidation of reduced vitamin K (or its analogue) by cytochromes, as was suggested by the model reactions proposed by Wessels [85], Harrison [116] and Clark *et al.* [117]. In the FMN pathway, an additional phosphorylating site can be readily envisaged in the span between TPN and cytochromes [94].

Further evidence, derived from fractionating chloroplasts, will be given in the next Section for the conclusion that cyclic photophosphorylation catalyzed by phenazine methosulphate probably proceeds by way of a "bypass" and is less dependent on enzymic chloroplast constituents than photosynthetic phosphorylation catalyzed by either vitamin K or FMN.

9. Structural association of chlorophyll with the photophosphorylating system

In photosynthetic bacteria the photophosphorylating system is structurally bound to chlorophyll in the smallest particles that function as units in the absorption of light energy, the chromatophores. Their analogues in green plants are the grana and it was of interest, therefore, to determine whether in chloroplasts photophosphorylation is indeed localized in the grana.

Photosynthetic phosphorylation was first observed in intact chloroplasts (Fig. 2) but experiments with disrupted chloroplasts soon demonstrated that structural integrity was not essential for this process. When whole chloroplasts were broken, active photophosphorylation systems were reconstituted by a recombination of chloroplast fractions and added cofactors [38, 39]. This technique proved effective in investigating the mechanism of photophosphorylation but provided no rigid evidence that the site of photophosphorylation is in the grana.

Direct evidence for the localization of photosynthetic phosphorylation in grana, freed from other chloroplast fractions, was obtained by Müller *et al.* [118] who prepared purified grana by sonication of isolated whole chloroplasts followed by a density gradient centrifugation technique. The purity of the grana obtained by these methods was determined by examination of electron micrographs of freeze-dried and air-dried grana preparations (Figs. 11 and 12). The freeze-drying technique avoids artifacts resulting from chemical fixation and retains the natural shape of the particles [119]. Cyclic photophosphorylation by purified grana is shown in Table IV.

Table IV shows that, at the high light intensity at which cyclic photophosphorylation by purified grana was measured, the highest rates were obtained in the system catalyzed by phenazine methosulphate. Photophosphorylation in this system was not increased by the addition of an aqueous chloroplast extract. In contrast, photophosphorylation catalyzed



FIG. 11. Electron micrograph of isolated spinach grana prepared for electron microscopy by a freeze-drying technique. 1 cm.² of leaf surface is estimated to have about 50 million chloroplasts. Whole chloroplasts were disrupted by a sonic vibration treatment for 10 sec. The grana were isolated in sucrose by a density gradient centrifugation technique and the sucrose removed by washing with 2% NaCl. The grana were used in electron microscopy involving a modified freeze-drying technique [119] that avoids possible artifacts resulting from chemical fixation and retains the natural shape of particles. Magnification: 59 000 \times (Müller, Steere, and Arnon, [118]).

by vitamin K or FMN proceeded at a lower rate and was markedly increased by the addition of chloroplast extract; however, even with this increase, it failed to reach the rate of photophosphorylation in the phenazine methosulphate system.

These results support the conclusion that grana are the site of the "primary" photophosphorylation reaction (equation (3)), the one that is

visualized as occurring between the "terminal" cytochrome and chlorophyll and is shown in Fig. 4 as being catalyzed by phenazine methosulphate. (Significant in this connection are the recent findings of James and Leech that, like the bacterial cytochromes in chromatophores, the

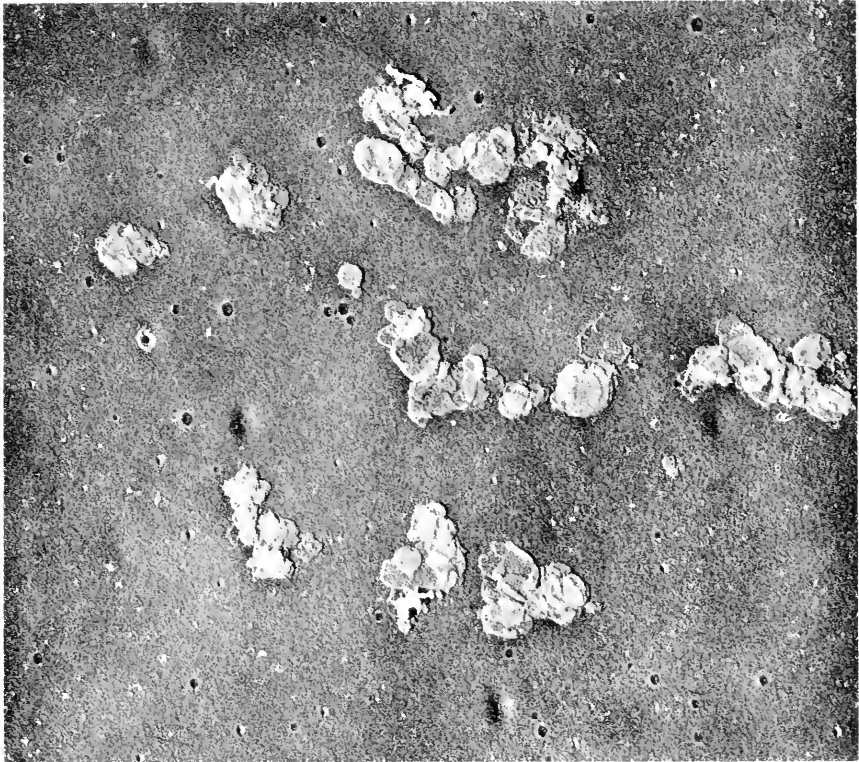


FIG. 12. Electron micrograph of isolated spinach grana fixed in formaldehyde. Whole chloroplasts were disrupted by sonic vibration treatment for 10 sec. The grana were isolated by differential centrifugation and filtration through a double layer of Whatman No. 2 filter paper. The isolated grana were fixed for 2 hr. in 6% formaldehyde at pH 6.5. A comparison with similar particles prepared by the freeze-drying technique (Fig. 11) shows that they collapsed in the formaldehyde treatment. The background in the formaldehyde treatment is free from salt (compare with Fig. 11). Magnification: 10 000 \times (Müller, Steere, and Arnon [118]).

chloroplast cytochromes, *f* and *b*, are "entirely confined to the grana" [120]). At high light intensity the "bypass" pathway catalyzed by phenazine methosulphate gives high rates of photophosphorylation because it is not limited by the absence of chloroplast constituents that lie outside the grana. The beneficial effect of added chloroplast extract on the FMN

TABLE IV

CYCLIC PHOTOPHOSPHORYLATION BY PURIFIED GRANA WITH AND WITHOUT ADDED CHLOROPLAST EXTRACT, ILLUMINATION 35 000 LUX

(Müller, Steere, and Arnon [118])

Treatment	$Q_p^{\text{chl}*}$
Phenazine methosulphate	157
Phenazine methosulphate + chloroplast extract	145
Vitamin K ₃	39
Vitamin K ₃ + chloroplast extract	78
FMN	46
FMN + chloroplast extract	69

* Micromoles orthophosphate esterified per mg. chlorophyll per hour.

and vitamin K systems (Table IV) suggests that the extract contains some chloroplast constituents that are involved in these pathways but not in the pathway catalyzed by phenazine methosulphate.

The close structural association, in both chloroplasts and bacterial chromatophores, of the phosphorylating activity with the chlorophyll pigments suggests that the harnessing of light energy in photosynthesis is more closely associated with ATP formation than with CO₂ assimilation. The enzymes responsible for CO₂ assimilation are easily dissociable from grana† in the case of chloroplasts [38, 39, 37], and not even structurally joined together in the case of bacterial chromatophores [68, 121]. These facts are in agreement with the view [94, 95] that in the course of biochemical evolution, photosynthesis first emerged as a process for converting light energy into ATP and this "primitive" photosynthesis became only later a process linked to CO₂ reduction.

10. Cyclic photophosphorylation as primitive photosynthesis

In the conventional view of photosynthesis, the chemical energy obtained by the conversion of absorbed light is always used for the reduction of CO₂. The case that cyclic photophosphorylation is a "primitive" photosynthesis in the evolutionary sense, would therefore be strengthened, if examples could be found today of cases in which the contribution of light to carbon assimilation could be experimentally limited to the formation of ATP.

† Grana, as contrasted with whole chloroplasts, cannot assimilate CO₂ to the level of carbohydrates but retain a capacity for photochemical oxygen evolution and photosynthetic phosphorylation. These findings do not exclude the *catalytic* participation of CO₂ in the mechanism of oxygen evolution as has recently been proposed by Warburg *et al.* (*Z. Naturf.* **14b**, 712-724, 1959).

Two such cases of photosynthesis in *Chromatium* have recently been described by Losada *et al.* [121]. In one case the sole source of carbon was acetate and in the other, CO₂. The photoassimilation of acetate occurred in the absence of an external hydrogen donor whereas in the photoassimilation of CO₂ the reductant was exogenous hydrogen gas. The sole contribution of light in both cases was the formation of ATP.

In the photoassimilation of CO₂, ATP was required for the formation

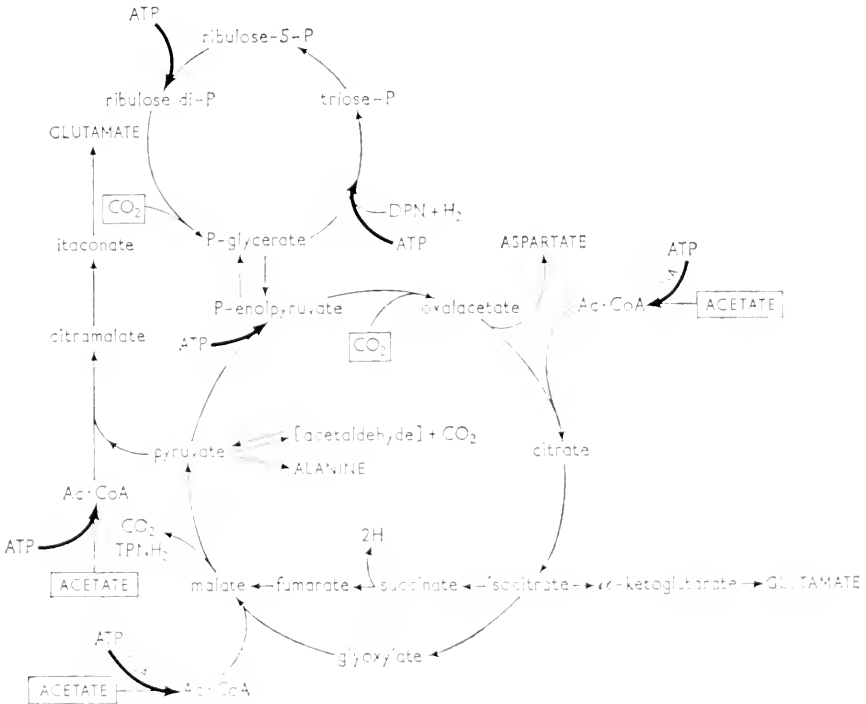


FIG. 13. Reactions of carbon assimilation in *Chromatium*. Further details are given by Losada, Trebst, Ogata, and Arnon [121].

of an activated intermediate (ribulose diphosphate, phosphoenolpyruvate, or 1,3-diphosphoglycerate) for a subsequent carboxylation or reduction, whereas in the photoassimilation of acetate, ATP was required for the activation of the carbon source itself, by forming acetyl-CoA from acetate and coenzyme A. The activated compounds then become ready for participation in the synthetic reactions that are catalyzed by specific enzyme systems, all of which function in the dark. A summary of the reactions of ATP, that have now been experimentally documented [121] in the carbon metabolism of *Chromatium*, is given in Fig. 13.

Evidence that the sole contribution of light in these reactions is the

formation of ATP, was obtained by replacing light with a supply of exogenous ATP and finding that carbon assimilation would then proceed in the same manner in the dark as in the light [121]. Other evidence for the equivalence of light and ATP is given in Table V. Here assimilation occurred either in the dark with added ATP or in the light when ATP was allowed to form photosynthetically. If, however, the ATP formed in light was trapped by an added hexokinase-glucose system then acetate assimilation ceased. The addition of hexokinase alone, without glucose as the ATP acceptor, was not inhibitory (Table V).

TABLE V

EQUIVALENCE OF ATP AND LIGHT IN THE ASSIMILATION OF ^{14}C -ACETATE BY CELL-FREE PREPARATIONS OF *Chromatium*

(Losada, Trebst, Ogata, and Arnon [121])

Treatment	^{14}C Carbon fixed in soluble compounds (Thousands of counts/min)
1. Dark, control	27
2. Dark, ATP	180
3. Dark, ATP, hexokinase	186
4. Dark, ATP, hexokinase, glucose	6
5. Light, control	414
6. Light, hexokinase	348
7. Light, hexokinase, glucose	20

Each vessel included, in a final volume of 1.5 ml., cell-free extract, containing 0.3 mg. bacteriochlorophyll and the following in micromoles: tris buffer, pH 7.8, 80; cysteine, 20; magnesium chloride, 5; manganese chloride, 2; potassium chloride, 20; coenzyme A, 0.3; oxalacetate, 10; [^{14}C]-acetate, 3. 1.5 mg. hexokinase, type III (Sigma Chemical Co.), 10 μ moles glucose, and 4 μ moles ATP were added as indicated. In treatment 5, 6 and 7 no addition of ADP was necessary to supplement the catalytic amounts present in the cell-free extracts.

The experimental substitution of ATP for light was considered particularly significant because it was found in photosynthetic bacteria such as *Chromatium*, that are unique in the living world in being strict phototrophs. *Chromatium*, unlike, for example, *Chlorella* or photosynthetic bacteria of the genus *Rhodospirillum*, cannot replace its light-dependent mode of life by a heterotrophic, aerobic metabolism in the dark [122, 123, 124]. *Chromatium* grows only in the light [122, 123], and being an obligate anaerobe, does not possess an alternative way for forming ATP by the mechanism of oxidative phosphorylation.

As regards the photoassimilation of acetate in another photosynthetic bacterium, the facultative anaerobe *R. rubrum*, a similar view that the

contribution of light is limited to cyclic photophosphorylation was recently expressed, on the basis of independent evidence, by Stanier *et al.* [125].

In certain circumstances, ATP formation may be the sole contribution of the photosynthetic process, not only in bacteria but also in higher plants. We have suggested elsewhere [95] that in green plants cyclic photophosphorylation may continue forming ATP when CO₂ assimilation is, for one reason or another, reduced or even stopped altogether. This might arise during the well-known midday closure of stomata in leaves of higher plants [126, 127] which restricts the supply of CO₂. The closure of stomata often coincides with an abundance of starch and an incipient water deficit in the photosynthesizing cells. Under these conditions cyclic photophosphorylation, which consumes neither CO₂ nor water, would be a useful device for generating ATP to drive the many ATP-dependent reactions, notably the synthesis of polysaccharides, proteins and fats.

These theoretical deductions for higher plants have recently received experimental support from the work of Maclachlan and Porter [128]. They reported the first known instance of utilization of light energy in leaf tissue for the synthesis of starch from labelled glucose, under conditions when CO₂ assimilation was excluded but cyclic photophosphorylation could proceed.

II. Pyridine nucleotide reduction by hydrogenase in the dark

In the examples of photosynthesis in which the contribution of light was limited to ATP formation, no reductant was needed in the conversion of glucose to starch in leaves. In the assimilation of acetate by bacteria, hydrogen is released for metabolic purposes and no additional hydrogen donor is required [121]. But the assimilation of CO₂ requires in addition to ATP, a supply of a reductant, i.e. reduced pyridine nucleotide. It was stated earlier that in photosynthesis of green plants both of these components of assimilatory power are formed at the expense of light energy. It is necessary, therefore, to trace the transition from a primitive photosynthesis in which light is used only for the formation of ATP to the "advanced" type of photosynthesis, observed in green plants, in which light energy is used not only for ATP formation but also for the reduction of pyridine nucleotide and the simultaneous evolution of oxygen.

In the photoassimilation of CO₂ by *Chromatium* the added reductant was hydrogen gas [129]. This is the simplest reductant usable by living cells. Cell-free hydrogenases from non-photosynthetic bacteria are known to reduce pyridine nucleotides with molecular hydrogen [130, 131; cf. 40].

From the standpoint of photosynthesis, it was important to know if the hydrogenases of photosynthetic bacteria could also reduce pyridine nucleotide with molecular hydrogen in the *dark*, since this would provide a mechanism, independent of light, for the formation of the reductant for CO₂ assimilation. In photosynthetic bacteria, the only cell-free hydrogenase tested in this respect, that of *R. rubrum*, was reported to be unable to reduce acceptors with potentials less than 0 volts [132] which would thus exclude pyridine nucleotides ($E'_0 = -0.32$ V.).

TABLE VI

PYRIDINE NUCLEOTIDE REDUCTION WITH MOLECULAR HYDROGEN BY CELL-FREE PREPARATIONS OF *Chromatium*

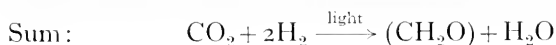
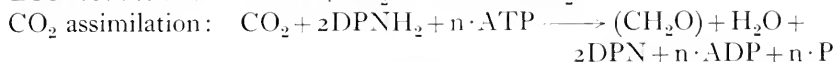
(Ogata, Nozaki, and Arnon [91])

Treatment	DPN Series		TPN Series	
	Light	Dark	Light	Dark
Pyridine nucleotide (PN)	0.08	0.09	0.07	0.07
Benzyl viologen (BV)	-0.08	-0.08	-0.08	-0.08
PN + BV	0.80	0.86	0.33	0.42

Each vessel included, in a final volume of 3.0 ml., a cell-free preparation (PS) containing 0.3 mg. bacteriochlorophyll and the following in micromoles: tris buffer, pH 7.8, 80; MgCl₂, 5; potassium phosphate, 5; KCl, 50; and when added, DPN, 4; TPN, 4; and benzyl viologen, 0.1 (a gift of Dr. H. Gest). 0.1 ml. of 20% KOH was present in the centre well. The reaction was carried out in an atmosphere of hydrogen at 25°. Illumination, when given, was 35 000 Lux. At the end of the reaction, an aliquot of the reaction mixture was precipitated with saturated (NH₄)₂SO₄, pH 8, centrifuged and the optical density of the clear supernatant fluid was measured at 340 m μ .

The subject was reinvestigated by Ogata *et al.* [91], using the cell-free hydrogenase of *Chromatium*. As in other photosynthetic bacteria (for example, *R. rubrum* [132]), an active hydrogenase was also found in *Chromatium*. The *Chromatium* hydrogenase reduced DPN and TPN with molecular hydrogen in the dark in the presence of benzyl viologen. The enzyme was more active toward DPN than TPN (Table VI).

These results indicated that in the presence of hydrogen gas, *Chromatium* cells do not require light for the reduction of pyridine nucleotides. The role of light is then limited to ATP formation, without which CO₂ assimilation cannot occur [121]. Photosynthesis by *Chromatium* in the presence of molecular hydrogen may, therefore, be summarized as follows:

Light phase*Dark phase*

Several algal species are known to contain hydrogenases and to acquire, after adaptation to hydrogen, a capacity to photoassimilate CO₂ with the aid of molecular hydrogen [133, 134, 135]. This process, which Gaffron named photoreduction [134], appears to be the same type of photosynthesis as that in *Chromatium* when it is supplied with hydrogen gas. It seems likely that photoreduction by algae is a case of reversion to a primitive photosynthesis of an earlier epoch when hydrogen gas was present in the environment and the sole contribution of light was the formation of ATP by cyclic photophosphorylation.

12. The photoreductant in bacteria

Although photosynthetic bacteria when supplied with hydrogen gas do not require light energy for the production of DPNH₂ (or TPNH₂), a different situation arises when photosynthetic bacteria are grown with such hydrogen donors as succinate or thiosulphate [122, 123]. Electrons donated by these substances have an insufficient reducing potential for reducing DPN (or TPN) in the dark.

Additional energy is then required to bring about the reduction of DPN (or TPN) and, in a photosynthetic mode of life without oxygen which is characteristic of photosynthetic bacteria, this additional energy must come from light. If the electron flow mechanism is fundamental to the conversion of light into chemical energy, how can it apply to the photoreduction of pyridine nucleotides by thiosulphate or succinate?

An attractive hypothesis was to consider bacterial photosynthesis with thiosulphate and succinate as an extension of bacterial photosynthesis with hydrogen gas, when the photochemical events proper are restricted to the formation of ATP by cyclic photophosphorylation. The primary photochemical act that results in the generation by the excited chlorophyll of a high energy electron and of the ultimate electron acceptor, [Chl⁺], would be the same in both cases. But in the thiosulphate and succinate type of

bacterial photosynthesis, not all of the high energy electrons would return via the cyclic route to $[\text{Chl}^+]$. Some of them would be passed on to pyridine nucleotide and used for CO_2 assimilation.

The electrons so removed from the photoreceptor particle would be replaced by electrons donated by thiosulphate or succinate. This electron transfer would be mediated by cytochromes. Thiosulphate and succinate would thus act as hydrogen donors that reduce bacterial cytochromes after these are oxidized by chlorophyll in light. The cytochrome system in photosynthetic bacteria would be a gateway for the entry of electrons of a

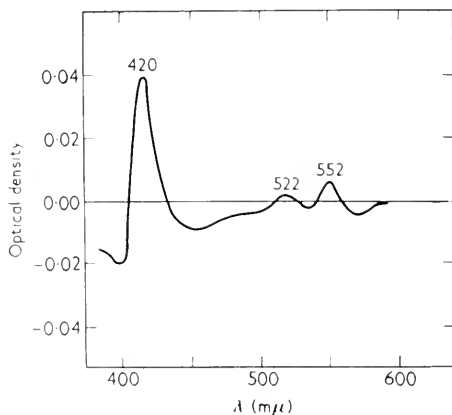


FIG. 14. Reduction of *Chromatium* cytochromes by thiosulphate in a cell-free system. Reaction mixture included, in a final volume of 3.0 ml. of 0.1 M tris buffer, pH 7.8, chromatophores (P) containing 0.1 mg. bacteriochlorophyll and supernatant fluid (S) corresponding to 0.3 mg. bacteriochlorophyll. 20 μ moles of thiosulphate were added to one of a pair of Thunberg-type cuvettes and the resulting difference spectrum was measured in a Cary spectrophotometer after 20 min. at room temperature. Gas phase, argon (Losada, Nozaki and Arnon [136]).

relatively low reducing potential and for their transfer to chlorophyll, where they would be raised to a higher reducing potential at the expense of the energy of absorbed light.

The proposed sequence of reactions in photosynthetic bacteria will be collectively designated as the non-cyclic electron flow mechanism. The three components of the non-cyclic electron flow mechanism are (a) an external electron donor system (represented here by thiosulphate or succinate), (b) the photoreceptor particle which raises the donated electron to a higher reducing potential at the expense of the energy of light, and (c) the electron acceptor system (exemplified by DPN or TPN).

Experimental support for the non-cyclic electron flow mechanism in bacterial photosynthesis has recently become available. First, it was

established that *Chromatium* particles have enzymes catalyzing the transfer of electrons from thiosulphate and succinate to cytochromes [136, 1]: the reduction of oxidized *Chromatium* cytochromes by thiosulphate is shown in Fig. 14 and by succinate in Fig. 15. Second, illuminated photosynthetic bacteria reduce pyridine nucleotides in the presence of succinate or some other electron donor that is less reduced than pyridine nucleotides. The photoreduction of DPN was observed by Frenkel [146] and Vernon and

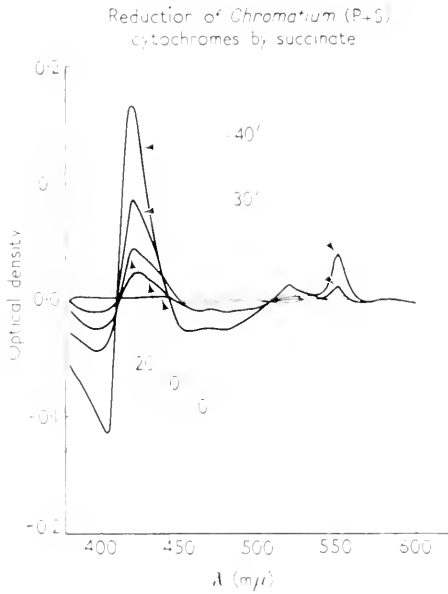


FIG. 15. Reduction of *Chromatium* cytochromes by succinate in a cell-free system. Reaction mixture included, in a final volume of 0.3 ml. of 0.2 M tris buffer, pH 7.8, chromatophores (P) containing 0.06 mg. bacteriochlorophyll and supernatant fluid (S) corresponding to 0.3 mg. bacteriochlorophyll. 10 μ moles of succinate was added to one of a pair of cuvettes and the resulting difference spectrum was measured in a Cary spectrophotometer at the indicated time intervals (Nozaki, Ogata, and Arnon [114]).

Ash [147] in *R. rubrum* and by Ogata *et al.* in *Chromatium* [91]. In more recent experiments we have found that in the presence of succinate and light, unwashed chromatophores from *R. rubrum*, unaided by enzymes from chloroplasts (cf. [147]) reduce both di- and triphosphopyridine nucleotide.

Additional support for the non-cyclic electron flow mechanism in bacterial photosynthesis has come from recent experiments on the photo-production of hydrogen gas and photofixation of nitrogen gas. We found

in *Chromatium* a light-dependent transfer of electrons from thiosulphate or succinate not only to pyridine nucleotides but also to H^+ and N_2 . The transfer of electrons to H^+ , a reaction that is catalyzed by hydrogenase, results in photoproduction of hydrogen gas. The transfer of electrons to N_2 constitutes photofixation of N_2 . These light reactions will now be discussed in more detail.

PHOTOPRODUCTION OF HYDROGEN GAS

Figure 16 illustrates a vigorous photoproduction of molecular hydrogen from thiosulphate [136] and Fig. 17 shows photoproduction of molecular

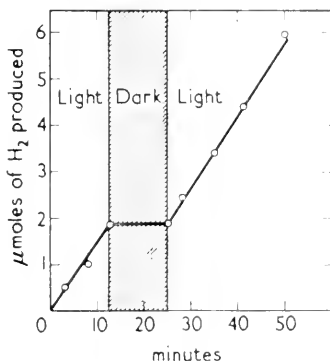


FIG. 16. Light-dependent evolution of hydrogen gas from thiosulphate by *Chromatium* cells. The reaction mixture included 0.1 g. of washed cells, suspended in 2.6 ml. of a modified nutrient solution from which nitrogen compounds were omitted, 0.3 ml. of 0.5 M tris buffer, pH 7.2, and 0.1 ml. of 0.2 M sodium thio-sulphate. 0.1 ml. of 20% KOH was placed in the centre wells of the Warburg manometer flasks. The reaction was run at 30°. Gas phase argon. Illumination 50 000 Lux (Losda, Nozaki, and Arnon [136]).

hydrogen from succinate [1]. In both cases the evolution of hydrogen occurred in the presence of KOH and seemed to be independent of CO_2 assimilation. Gas evolution ceased when light was turned off and resumed when light was turned on again. The evolved gas was identified as hydrogen (Table VII) by adsorption on palladium asbestos [137]. Photoproduction of H_2 was inhibited by carbon monoxide (Table VII).

The evolution of hydrogen by illuminated *Chromatium* cells showed a marked pH dependence (Fig. 18). The reaction was most vigorous at the more acid pH. Little hydrogen was evolved at pH 8.0.

Similar to the inhibition of hydrogen evolution in the presence of organic hydrogen donors [138, 139], photoproduction of hydrogen from

TABLE VII

PHOTOPRODUCTION OF HYDROGEN FROM SUCCINATE BY *Chromatium*
(Ogata, Nozaki, and Arnon [91])

Treatment	$\mu\text{moles H}_2$ evolved
1. Complete	8.6
2. Complete, KOH omitted	8.2
3. Complete, dark	1.5
4. Complete, succinate omitted	0.6
1. Complete	5.9
2. Complete, succinate omitted	0.7
3. Complete, plus palladium asbestos	0.3
4. Complete, plus carbon monoxide	-0.7

The complete system contained, in a final volume of 3.0 ml., 100 mg. wet cells that were suspended in a modified nutrient solution with nitrogen omitted, and the following in micromoles; tris buffer, pH 7.2, 80; MgCl_2 , 5; succinate, 20. 0.1 ml. of 20% KOH was present in the centre well. The reaction was carried out for 2 hr. at 30° in argon. Illumination 35 000 Lux.

thiosulphate was also inhibited by molecular nitrogen and ammonium ions. The results are shown in Fig. 19.

The photoproduction of hydrogen was dependent on the concentration of thiosulphate and was abolished by heating the cells (Fig. 20). Growing *Chromatium* cells were found, by analysis to have oxidized in 4 days 27 millimoles of added thiosulphate into 54 millimoles of sulphate. During

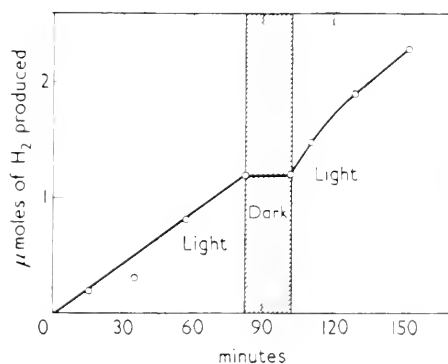


FIG. 17. Photoproduction of hydrogen gas from succinate by *Chromatium* cells. Experimental conditions as described in Table VII (Ogata, Nozaki, and Arnon [91]).

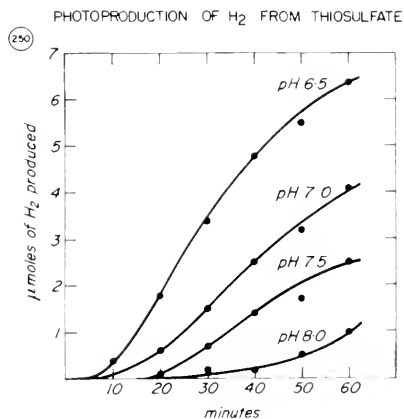


FIG. 18. Effect of pH on photoproduction of hydrogen gas from thiosulphate by *Chromatium* cells. Experimental conditions as described for Fig. 16. Phosphate buffer was used at pH 6.5 and 7.0 and tris buffer at pH 7.5 and 8.0 (Losada, Nozaki, and Arnon [136]).

this period the appearance of the culture indicated a transitional formation of elemental sulphur. The results are in agreement with the following sequence of reactions, in which thiosulphate is the donor of electrons that are activated by light and used either for the assimilation of carbon and

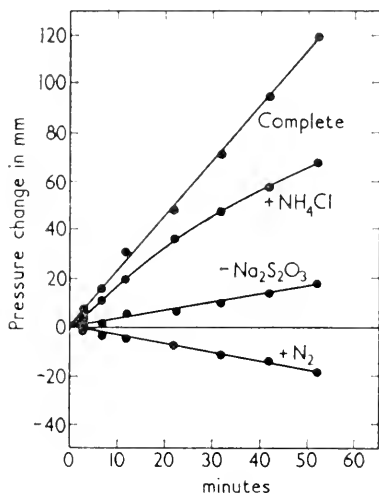
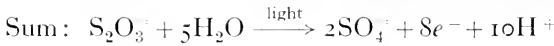
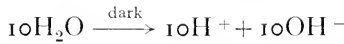
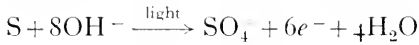
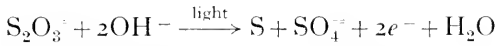


FIG. 19. Effect of N₂ and NH₄Cl on photoproduction of H₂ from thiosulphate by *Chromatium* cells. Experimental conditions as described for Fig. 16. 5 μmoles NH₄Cl and N₂ gas were used as indicated (Losada, Nozaki, and Arnon [136]).

nitrogen into cellular substance or for the photoproduction of hydrogen gas.



The photoproduction of hydrogen gas approached the stoichiometry of the first reaction, i.e. for one molecule of thiosulphate used, two electrons and two protons were combined with the aid of hydrogenase and evolved as H₂.

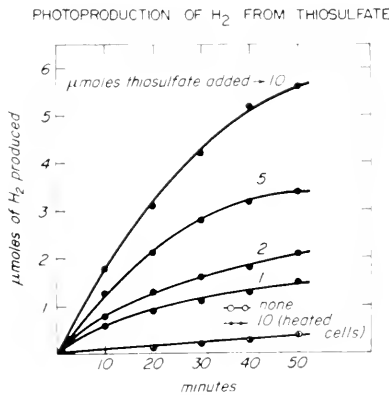
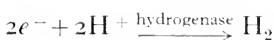
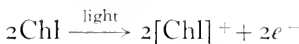
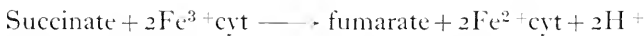


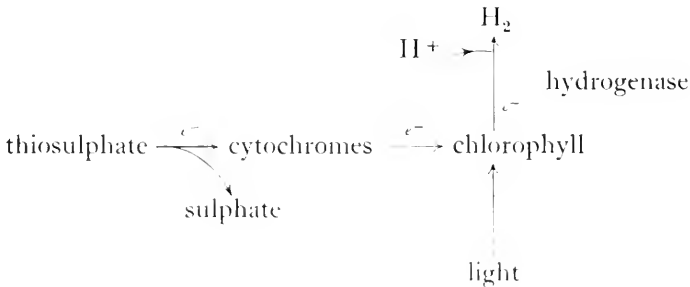
FIG. 20. Photoproduction of hydrogen gas by *Chromatium* cells as a function of added thiosulphate. The control treatment contained cells that have been heated at 100° for 10 min. Other experimental conditions are described for Fig. 16 (Losada, Nozaki, and Arnon [136]).

With succinate as the electron donor, the proposed sequence of reactions which results in the photoproduction of hydrogen is represented as follows:



The results with thiosulphate provided the first experimental evidence for a light-dependent hydrogen evolution from an *inorganic* electron donor by a photosynthetic organism [136, cf. 143]. Photoproduction of hydrogen was first observed in algae by Gaffron and Rubin [140] and in photosynthetic bacteria by Gest and Kamen [138]. In algae the photoproduction of hydrogen seemed to depend on internal electron donors of metabolic origin [140] whereas photoevolution of hydrogen by photosynthetic bacteria appeared to depend on exogenous organic acids and CO_2 [138, 141, 142, 143, 139, 144, 145].

The evolved hydrogen has previously been ascribed to photodecomposition of water [140, 145] or to decomposition of α -ketoglutaric acid [139]. We regard the photoproduction of molecular hydrogen from thiosulphate (or succinate) as evidence for a "non-cyclic" electron flow mechanism in bacterial photosynthesis as depicted below:



PHOTOFIXATION OF NITROGEN GAS

Nitrogen fixation by photosynthetic organisms [149, 141, 139, 150-153] may also be viewed as resulting from a non-cyclic electron flow in which electrons pass from an external electron donor, via cytochromes, to chlorophyll excited by light, and thence to molecular nitrogen.

This interpretation was substantiated by using thiosulphate and succinate as electron donors for fixation of nitrogen gas by illuminated *Chromatium* cells [154, 155]. Figure 21 shows photofixation of nitrogen gas with thiosulphate as the electron donor and Fig. 22 shows photofixation of nitrogen with succinate as the electron donor. In both cases, *Chromatium* cells fixed N_2 only in light. Fixation ceased when the light was turned off and resumed when the light was turned on again. The dependence of N_2 fixation on light and an external electron donor, was confirmed with the use of ^{15}N isotope* (Table VIII).

In the case of thiosulphate, photofixation of N_2 was greatly increased by the addition of oxaloacetate which probably acted as an amino group

* We are indebted to Dr. C. C. Delwiche for the determinations of ^{15}N .

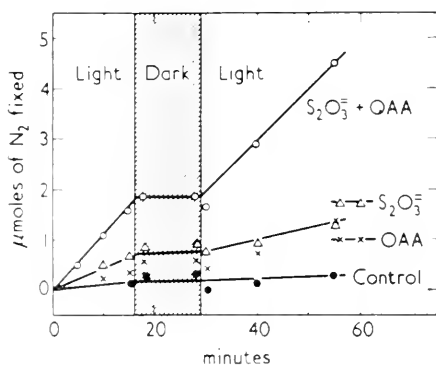


FIG. 21. Effect of thiosulphate and oxaloacetate on photofixation of nitrogen gas by *Chromatium* cells. The reaction mixture included in a final volume of 2.0 ml., 0.1 g. of washed cells, suspended in a modified nutrient solution, pH 7.2 (from which nitrogen compounds were omitted). 20 μ moles each of thiosulphate and oxaloacetate were added as indicated. 0.1 ml. of 20% KOH was placed in the centre wells of the Warburg manometer flasks. The reaction was run at 30°. Gas phase, nitrogen. Illumination 50 000 Lux (Losada, Nozaki, Tagawa, and Arnon [155, 154]).

acceptor. Succinate served both as electron donor and as source of a carbon skeleton that is needed for accepting an amino group.

As shown in Fig. 23, photofixation of N_2 with thiosulphate was inhibited by ammonia (cf. [149]). However, neither ammonia nor nitrogen gas inhibited CO_2 fixation by illuminated *Chromatium* cells when thio-

TABLE VIII

EFFECT OF LIGHT AND ELECTRON DONORS ON FIXATION OF MOLECULAR NITROGEN BY *Chromatium* CELLS

(Losada, Nozaki, Tagawa, and Arnon [155, 154])

Treatment	Atom percent ^{15}N excess
Dark, succinate	0.004
Dark, thiosulphate + oxaloacetate	0.003
Light	0.288
Light, succinate	1.049
Light, thiosulphate + oxaloacetate	1.467

40 μ moles each of thiosulphate, oxaloacetate and succinate were added as indicated. Other experimental conditions as in Fig. 21, except that no KOH was included, and the gas atmosphere was 90% argon and 10% N_2 containing 30 atom percent excess ^{15}N . Reaction time was 2 hr.

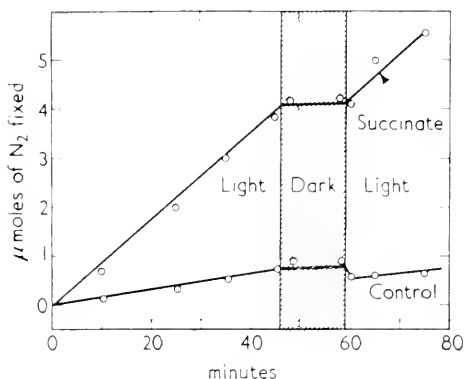


FIG. 22. Effect of succinate on photofixation of nitrogen gas by *Chromatium* cells. Experimental conditions as described in Fig. 21, except that thiosulphate and oxaloacetate were omitted. 20 μ moles of succinate were added as indicated (Losada, Nozaki, Tagawa, and Arnon [155, 154]).

sulphate was the electron donor (Table IX). These results suggest that ammonia or N_2 did not inhibit the flow of electrons that are required for CO_2 assimilation (by way of $DPNH_2$ or $TPNH_2$). At high light intensity, when the electron flux is large enough to cope with the requirements of

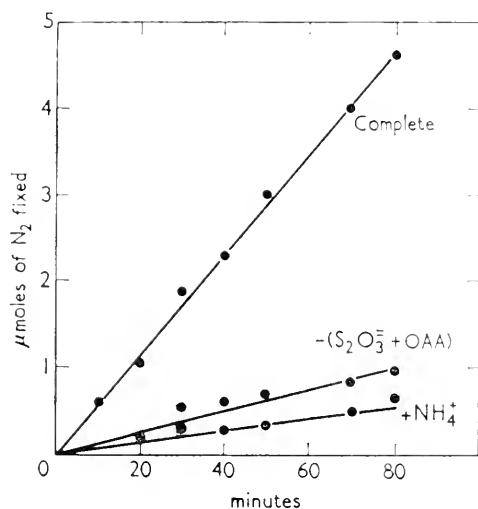


FIG. 23. Effect of ammonia on photofixation of nitrogen gas with thiosulphate by *Chromatium* cells. Experimental conditions were the same as described for Fig. 21 except that 30 μ moles of NH_4Cl were added as indicated (Losada, Nozaki, Tagawa, and Arnon [155, 154]). The addition of ammonia to, or the omission of thiosulphate and oxaloacetate from, the complete system prevents nitrogen fixation.

TABLE IX

EFFECT OF NITROGEN GAS AND AMMONIA ON CO₂ FIXATION BY ILLUMINATED *Chromatium* CELLS WITH THIOSULPHATE AS ELECTRON DONOR

(Losada, Nozaki, and Arnon [136])

Treatment	Total ¹⁴ C ₂ fixed counts min. (thousands)
Argon	524
Argon + thiosulphate	2190
N ₂ + thiosulphate	2238
Argon + thiosulphate + NH ₄ Cl	2436

The reaction mixture contained in a total volume of 3 ml.: 50 mg. of washed cells, suspended in 2.3 ml. of a modified nutrient solution from which nitrogen compounds were omitted, and the following in micromoles: tris buffer, pH 7.5, 150; thiosulphate, 20; and sodium bicarbonate labelled with ¹⁴C, 1.0. 30 μmoles of NH₄Cl were added as indicated. Gas phase, argon or N₂ as indicated. The reaction was carried out at 30°.

both N₂ fixation and CO₂ assimilation, both processes can occur simultaneously.

CELL-FREE NITROGEN FIXATION

In preliminary experiments, when cell-free *Chromatium* preparations were supplied in the dark with a mixture of hydrogen and nitrogen gas,

TABLE X

N₂ FIXATION IN THE DARK BY CELL-FREE EXTRACTS OF *Chromatium* SUPPLIED WITH HYDROGEN GAS

(Losada, Nozaki, Tagawa, and Arnon [155, 154])

Cofactors added	Gas uptake, mm. pressure change	
	Gas phase H ₂	Gas phase 50% H ₂ + 50% N ₂
None	0	-11
DPN	-22	-62

The reaction mixture contained, in a final volume of 3 ml.: cell-free extract containing 0.27 mg. bacteriochlorophyll, and the following in micromoles: tris buffer, pH 7.5, 150; MgCl₂, 5; and, where indicated, DPN, 0.5 (added jointly with benzyl viologen, 0.2). The gas phase was in one case hydrogen gas and in the other a mixture of nitrogen and hydrogen in equal volume. KOH was present in the centre wells of the manometer vessels. The reaction was run for 45 min. at 25° in the dark.

they absorbed more gas than in the control treatment in which the gas phase consisted solely of hydrogen (Table X). This seems to indicate that cell-free *Chromatium* preparations were fixing N_2 , with the aid of hydrogen as the electron donor—an interpretation that was strengthened by experiments with ^{15}N isotope (Table XI). These findings support the view that the role of light in photofixation of N_2 is to generate electrons with a reducing potential that is at least equal to that of H_2 . When H_2 was supplied in the gas phase, light was no longer necessary for the fixation of N_2 . It seems that pyridine nucleotides mediate the reduction of N_2 (Tables X and XI). However, the experiments on N_2 fixation by cell-free *Chromatium* preparations are at an early stage and the drawing of final conclusions would be premature.

TABLE XI

$^{15}N_2$ FIXATION IN THE DARK BY CELL-FREE EXTRACTS OF *Chromatium*
(Losada, Nozaki, Tagawa, and Arnon [155, 154])

No.	Reductant added	^{15}N atom per cent excess	
		Experiment A	Experiment B
1	None	0.0255	0.0274
2	DPNH ₂	0.0480	—
3	H ₂	0.0362	0.0430

The reaction mixture contained in a final volume of 3 ml.: cell-free extract, containing 0.3 mg. bacteriochlorophyll, and the following in micromoles: tris buffer, pH 7.8, 100; $MgCl_2$, 5; benzyl viologen, 0.2. Treatments 1 and 3 received 0.5 μM DPN, and Treatment 2, 1 μM DPNH₂. All vessels received 0.5 atmosphere nitrogen containing 96 atom-% excess ^{15}N . Treatment 3 received in addition 0.5 atmosphere hydrogen gas. The experiment was run at 25° for 2 hr. in the dark.

To recapitulate, the photofixation of N_2 and the photoproduction of H_2 , from electron donors such as thiosulphate or succinate, are taken as evidence for a non-cyclic electron flow mechanism, that supplements the cyclic mechanism for ATP production. Preliminary experiments indicate that the non-cyclic electron transport in *Chromatium* that results in pyridine nucleotide reduction is coupled with the formation of ATP [155].

A diagrammatic representation of the proposed non-cyclic electron flow mechanism in photosynthetic bacteria is shown in Fig. 24. Three of the external electron acceptors have now been identified: pyridine nucleotides, nitrogen gas, and protons. It seems likely that protons serve as electron acceptors and hydrogen gas is evolved when electrons activated by light become surplus, i.e. when they are not consumed in metabolic

TABLE XII

HYDROGEN EVOLUTION FROM REDUCED METHYL VIologen BY CELL-FREE HYDROGENASE FROM *Chromatium*

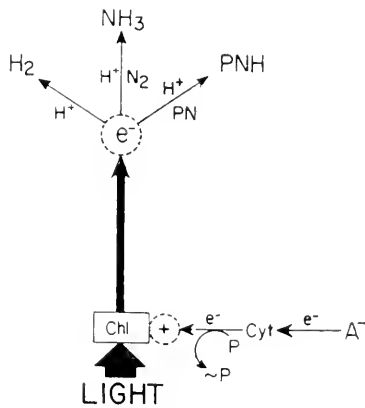
(Ogata, Nozaki, and Arnon, [91])

	μmoles H ₂ evolved 10 min./mg. chl.
Complete system	12.8
Methyl viologen omitted	none
Na ₂ S ₂ O ₄ omitted	none
Hydrogenase omitted	none

Complete system contained, in a final volume of 3.0 ml., cell-free suspension (PS) containing 0.4 mg. bacteriochlorophyll and 80 μmoles tris buffer, pH 7.2. 0.1 ml. of 20% KOH was present in the centre well and 16 μmoles of methyl viologen was added to the sidearm. Methyl viologen was reduced by adding Na₂S₂O₄ to the same sidearm while gassing with argon. The reaction was carried out at 30° in the dark.

reactions as in the reduction of CO₂ via pyridine nucleotides or in the photofixation of N₂.

The hydrogenase present in *Chromatium* particles that catalyzes hydrogen evolution in the light can also catalyze hydrogen gas evolution



Non-cyclic electron transport in *Chromatium*

FIG. 24. Scheme for non-cyclic electron flow in *Chromatium*. Details in the text.

in the dark (Table XII) when electrons are supplied at a sufficiently reducing potential, as for example by hydrosulphite (cf. [132]). Methyl viologen was required as a catalyst in this reaction (compare [156, 157]).

13. The photoreductant in plants: non-cyclic photophosphorylation

Photosynthetic bacteria can reduce pyridine nucleotide either with molecular hydrogen in the dark or with a less reduced electron donor, organic or inorganic, in the light. Green plants do not ordinarily contain hydrogenase, hence they cannot use hydrogen gas during photosynthesis for reducing pyridine nucleotide in the dark. They use water as the electron donor. The reduction of pyridine nucleotides with electrons donated by water requires a considerable input of energy which in photosynthesis is supplied by light.

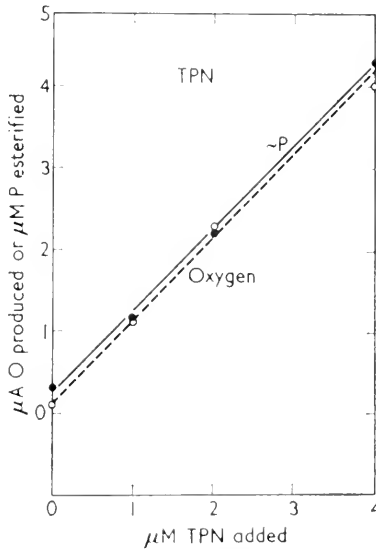
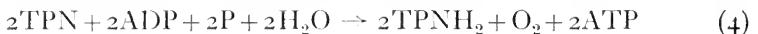


FIG. 25. Stoichiometry of oxygen evolution and ATP formation resulting from the photochemical reduction of TPN (Arnon, Whatley, and Allen, [95, 158]).

As already mentioned, isolated chloroplasts were known to reduce TPN in light with an accompanying evolution of oxygen [42, 43, 5]. This was regarded as a Hill reaction in which TPN served as the hydrogen acceptor. There was no evidence that this photochemical reduction of TPN was in any way linked with photosynthetic phosphorylation. Recently, however, the relation of photosynthetic phosphorylation to the photoreduction of TPN which at first seemed remote, was found to be direct [95, 104]. In the presence of ADP and orthophosphate (P), the photoreduction of TPN and oxygen evolution was coupled with the formation of ATP in accordance with equation (4).



Under appropriate experimental conditions [158] the evolution of one mole of oxygen was accompanied by the reduction of two moles of TPN, and the esterification of 2 moles of orthophosphate (Fig. 25). The stoichiometry of this reaction was the same when TPN was replaced by ferricyanide. With either TPN [159] or ferricyanide [95, 104] the rate of oxygen evolution is greatly increased when it is coupled with phosphorylation. The conventional Hill reaction could thus be viewed as an uncoupled photophosphorylation, i.e. a photochemical electron transport that is proceeding without its normally associated phosphorylation reaction.

It was proposed elsewhere [94, 1] that the reduction of TPN by chloroplasts in Reaction 4 involves a non-cyclic electron flow mechanism. Reaction 4 may thus be viewed as being analogous to the non-cyclic electron flow in bacteria (Fig. 24) and differing from it only in those

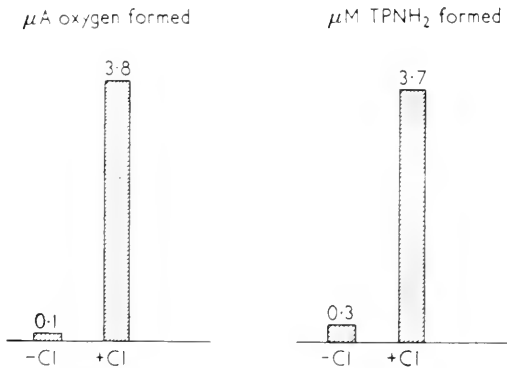


FIG. 26. Effect of chloride on reduction of TPN and evolution of oxygen. The reaction mixture contained in a volume of 3 ml. chloroplasts (P_{18}) containing 0.25 mg. chlorophyll; and the following, in micromoles: tris acetate buffer, pH 8.2, 80; TPN, 4; and a partly purified preparation of photosynthetic phosphopyridine nucleotide reductase. The plus chloride treatment received 10 μmoles KCl. Oxygen evolution was measured manometrically, and the TPNH_2 formed was measured by its absorption at 340 $m\mu$ (Bové, Bové, Whatley, and Arnon [103]).

aspects that reflect the special enzymic composition of chloroplasts. Unlike photosynthetic bacteria, chloroplasts contain neither N_2 -fixing enzymes nor hydrogenase. As a consequence, the electron acceptor end of the non-cyclic electron flow mechanism in chloroplasts can be coupled neither to photofixation of nitrogen nor to photoproduction of hydrogen gas, but only to CO_2 reduction (by way of TPNH_2).

The most characteristic difference between the non-cyclic electron flow mechanism of chloroplasts (equation (4)) and bacteria (Fig. 24) is in the electron donor system. In chloroplasts the electron donor is water (i.e. OH^-) whereas bacteria cannot use water but use inorganic or organic electron donors such as thiosulphate or succinate [94, 136].

This interpretation of the difference between the non-cyclic electron flow mechanism in chloroplasts and in photosynthetic bacteria is supported by recent evidence that it is experimentally possible to replace water as the electron donor in non-cyclic photophosphorylation by chloroplasts.

As was already mentioned (Section 7), photosynthetic reactions of chloroplasts in which oxygen is liberated require chloride, hence Reaction 4 could not proceed in the absence of chloride. As shown in Fig. 26, on omitting chloride from the reaction mixture (a step that included purification of those reagents that contained chloride impurities) TPN reduction and oxygen evolution ceased and photophosphorylation was abolished (Table XIII).

TABLE XIII

EFFECT OF CHLORIDE ON NON-CYCLIC PHOTOPHOSPHORYLATION BY ISOLATED CHLOROPLASTS

(Bové, Bové, Whatley, and Arnon [103])

Experiment	Electron acceptor	Micromoles ATP formed	
		- chloride	+ chloride
A	Ferricyanide	0.1	3.3
B	Ferricyanide	0.3	3.7
C	TPN	0.7	3.6
D	TPN	0.7	4.2

These results indicated that chloroplasts deprived of chloride cannot use water as the electron donor in Reaction 4. It was possible, however, that they could use other electron donors that did not involve an oxidation of water (i.e. OH^-) and a resultant oxygen evolution. Vernon and Zaugg [160] have found that chloroplasts which are incapable of photochemical oxygen evolution, retain the capacity for photoreduction of TPN with ascorbate (jointly with catalytic amounts of 2,6-dichlorophenol indophenol) as the electron donor.

Table XIV shows that using an ascorbate electron donor system, chloroplasts carried out a "bacterial" type of non-cyclic photophosphorylation in which ATP formation and TPN reduction were not accompanied by an evolution of oxygen. The participation of water (OH^-) as an electron donor was prevented here by the omission of chloride and also by the addition of dichlorophenyl dimethylurea as an inhibitor of oxygen evolution (cf. [160]).

These results support the view (cf. [121]) that the evolution of oxygen in non-cyclic photophosphorylation by chloroplasts (and hence in photosynthesis of green plants) is not fundamental to the key photosynthetic

TABLE XIV

NON-CYCLIC PHOTOPHOSPHORYLATION BY CHLOROPLASTS WITH ASCORBATE AS THE ELECTRON DONOR

(Whatley, Dieterle, and Arnon [161])

No.	Addenda	Effective electron donor	Oxygen evolved (μ atoms)	TPN reduced (μ moles)	ATP formed (μ moles)
1.	None	Water	3.0	3.4	2.4
2.	CMU	None	0	0.5	0.2
3.	CMU, ascorbate	Ascorbate	0	3.2	3.4

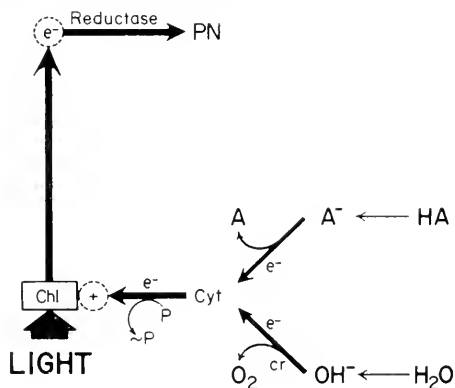
Treatment 1 contained: washed chloroplast fragments (P1S2), prepared in the absence of chloride, containing 0.5 mg. chlorophyll; 0.05 ml. purified spinach phosphopyridine nucleotide reductase, and the following in micromoles: tris/acetate buffer, pH 8.0, 40; $MgSO_4$, 5; $K_2H^{32}PO_4$, 10; ADP, 10; TPN, 4; and KCl, 10. In Treatment 2 KCl was omitted and 2×10^{-5} M *p*-chlorophenyl-dimethylurea (CMU) was added. Treatment 3 was the same as Treatment 2 except that 20 μ moles ascorbate and 0.2 μ mole 2,6-dichlorophenol indophenol were added (cf. [160]). The experiment was run for 20 min. at 15 (at a light intensity of 2000 foot candles).

The omission of chloride (Fig. 26) and the addition of CMU, a powerful inhibitor of oxygen evolution, prevented the use of water as an electron donor in the chloroplast system. Catalytic amounts of dichlorophenol indophenol served as an electron carrier [160] between ascorbate and the chloroplast system.

events, i.e. ATP formation and TPN reduction. Oxygen evolution occurs when water (OH^-), on donating an electron to the photosynthetic particle, becomes oxidized to oxygen. Under special experimental conditions, when ascorbate displaces water as the electron donor, no oxidation of OH^- occurs, only the oxidation of ascorbate [160]. This concept of the non-cyclic photophosphorylation in chloroplasts is represented in Fig. 27.

The proposed mechanism assigns to cytochromes a role in transporting electrons from the electron donor system to chlorophyll. Cytochromes are known to accept electrons from ascorbate but the suggestion that a photosynthetic cytochrome system mediates the transfer of electrons from OH^- to chlorophyll is put forward only as a working hypothesis [1]. This hypothesis implies that the chlorophyll-cytochrome complex must generate a sufficient oxidizing potential to drive the reaction $2H_2O \rightarrow O_2 + 4H^+ + 4e^-$, which at 25° and at pH 7, has $E'_0 = +0.815$ V. It might be argued that cytochrome *f*, the most oxidizing cytochrome now known to occur in chloroplasts, has a redox potential lower than oxygen [162], i.e. $E'_0 = +0.365$ V. However, it would be premature to conclude that our knowledge of redox potentials of cytochromes in chloroplasts is now complete.

The proposed reactions from OH^- to oxygen evolution appear to be thermodynamically feasible. The energy contribution of one einstein of red light, about 43 Kcal., is equivalent to a potential of 1.9 V. per faraday, and is sufficiently large, after making allowances for TPN reduction and ATP formation, to endow a chlorophyll-linked cytochrome with a redox potential more oxidizing than 0.815 V., as is needed for oxygen evolution.



Non-cyclic photophosphorylation (chloroplasts)

FIG. 27. Scheme for non-cyclic photophosphorylation in chloroplasts. Details in the text. Chloride is required for oxygen evolution.

It must be emphasized that, in our present state of knowledge, the proposed mechanism for oxygen evolution must remain tentative. The possibility exists that the transfer of electrons from OH^- to cytochromes requires an auxiliary input of light energy via a photosynthetic pigment.*

14. Oxygen-dependent cyclic photophosphorylation

The mechanisms of photosynthetic phosphorylation in chloroplasts discussed thus far include anaerobic cyclic photophosphorylation (Figs. 4 and 5) and non-cyclic photophosphorylation (Fig. 27). Recent work by Tsujimoto *et al.* [92] suggests the operation in chloroplasts of a third mechanism, an oxygen-dependent cyclic photophosphorylation.

As was already discussed in Section 4, a catalytic role for oxygen was envisaged in explaining the first experiments on photosynthetic phosphorylation, in which the presence of oxygen was required but no oxygen consumption was observed [13]. Interest in the role of oxygen was heightened when several laboratories reported that at low, "micro-catalytic", concentrations of FMN or vitamin K (Fig. 3), photophosphorylation remained dependent on oxygen [54-56].

* Note added in proof. Experimental evidence for a separate light reaction responsible for oxygen evolution has now been obtained. (cf. M. Losada, F. R. Whatley and D. I. Arnon, *Nature, Lond.* **190**, 606-610, 1961.)

From the standpoint of cellular physiology it was interesting to contrast the role of oxygen in ATP formation in photosynthesis with that in respiration. The participation of oxygen as the terminal electron acceptor in oxidative phosphorylation has conferred a marked superiority on respiration over fermentation, in the efficiency of converting the free energy of substrate into the energy of the pyrophosphate bonds of ATP. Was the efficiency of conversion of light energy into ATP also increased by the presence of oxygen?

To answer this question, photophosphorylation by chloroplasts was investigated in air and in nitrogen, at different concentrations of FMN or vitamin K, and particularly, at a *limiting light intensity*, when the efficiency

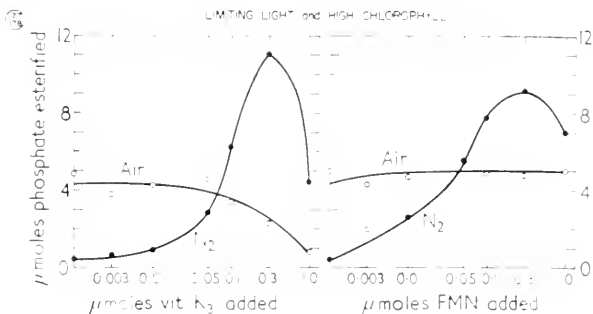


FIG. 28. Effect of FMN and vitamin K₃ concentration on cyclic photophosphorylation by spinach chloroplasts in nitrogen and air, at a low light intensity. The reaction mixture included, in a final volume of 3.0 ml., chloroplast fragments (C₁₈) containing 1.5 mg. chlorophyll; and in micromoles: tris buffer, pH 8.3, 80; MgSO₄, 5; K₂H³²PO₄, 15; ADP, 15; TPN, 0.3 (only in the FMN series). FMN or vitamin K₃ was added as indicated. The reaction was run for 30 min. at an illumination of 2 000 Lux (Tsujiyama, Hall, and Arnon, [92]).

of the energy conversion process could be best observed (compare Section 8). The results are shown in Fig. 28.

In limiting light, the highest rate of photophosphorylation was obtained in nitrogen at a concentration of approximately 10^{-4} M of either FMN or vitamin K. No photophosphorylation occurred in nitrogen without added cofactors but when these were added at an optimal concentration, the anaerobic system was about twice as efficient in converting light energy into ATP as the aerobic system.

The experiments represented by Fig. 28 were carried out with relatively high concentrations of chloroplast material. Under these conditions the aerobic system showed little increase in photophosphorylation from adding FMN or vitamin K. However, high concentrations of chloroplast material were found to be necessary to insure the effective operation of the anaerobic FMN system. The anaerobic vitamin K system functioned optimally at

lower concentrations of chloroplast material suggesting that it required less or fewer of the chloroplast factor(s) than were required for the anaerobic FMN system. These chloroplast factors for the FMN system appeared to be bound in the grana fraction and were not supplied by an aqueous extract of chloroplasts.

On comparing the aerobic and anaerobic systems under conditions when they responded optimally to the addition of cofactors, a marked difference was observed, depending on the presence or absence of oxygen, in the effect of two inhibitors, *o*-phenanthroline and CMU (*p*-chlorophenyldimethylurea) (a gift of Dr. C. E. Hoffman). The results are shown in Table XV.

TABLE XV

EFFECT OF *o*-PHENANTHROLINE (*o*-P) AND DICHLOROPHENYLDIMETHYLUREA (CMU) ON CYCLIC PHOTOPHOSPHORYLATION IN NITROGEN OR AIR

(Tsujiimoto, Hall, and Arnon [92])

Treatment	Percentage inhibition	
	CMU	<i>o</i> -P
Nitrogen, FMN	25	20
Nitrogen, vit. K ₃	19	27
Air, FMN	97	77
Air, vit. K ₃	85	64

In the nitrogen series the illumination was 2000 Lux for 30 min. and the reaction mixture included, in a final volume of 3 ml. chloroplast fragments (C₁₈) containing 1.5 mg. chlorophyll and 0.3 μ mole of FMN or vit. K₃. In the air series the illumination was 50 000 Lux for 5 min. and the reaction mixture included chloroplast fragments (C₁₈) containing 1 mg. chlorophyll and 0.003 μ mole of FMN or vitamin K₃. The final inhibitor concentrations were, 3×10^{-5} M for *o*-phenanthroline and 2×10^{-6} M for CMU. Other common components of the reaction mixture were, in micromoles: tris buffer, pH 8.3, 80; K₂H₃₂PO₄, 15; and MgSO₄, 5.

In agreement with findings of Wessels [54], Jagendorf and Avron [55] and Nakamoto *et al.* [56], *o*-phenanthroline and CMU, in the presence of air, inhibited photophosphorylation in the FMN and vitamin K systems. Relatively little inhibition by these two inhibitors was observed in an atmosphere of nitrogen. In other experiments, not reported here, phenazine methosulphate was found to differ from FMN and vitamin K in that its pathway was resistant to inhibition by *o*-phenanthroline and CMU, both in air and in nitrogen.

o-Phenanthroline and CMU are powerful inhibitors of oxygen evolution by illuminated chloroplasts (cf. [54-56]). It seems likely, therefore, that as was concluded earlier by Wessels [54] and Nakamoto *et al.* [56],

oxygen evolution is a component step in the "aerobic" photophosphorylation catalyzed by FMN or vitamin K, and that molecular oxygen, when present, acts as an electron acceptor in photosynthetic phosphorylation. This conclusion is supported by the observed effect of chloride on cyclic photophosphorylation with vitamin K and FMN in air and in nitrogen (Table XVI). The omission of chloride had scarcely an effect on photophosphorylation in nitrogen, but it severely inhibited photophosphorylation in air, which depends on the photochemical evolution of oxygen.

TABLE XVI

EFFECT OF CHLORIDE ON CYCLIC PHOTOPHOSPHORYLATION IN NITROGEN OR AIR
(Tsujiimoto, Hall, and Arnon [92])

Treatment	μ moles P esterified	
	- chloride	+ chloride
Nitrogen, FMN	5.1	5.7
Nitrogen, vit. K ₃	9.7	9.9
Air, FMN	0.5	6.1
Air, vit. K ₃	0.4	5.5

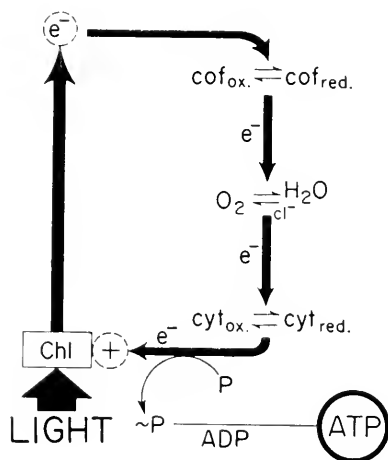
Experimental conditions as in Table XV, except that chloroplasts were prepared in 0.5 M sucrose and chloride-free reagents were used. 0.2 mg. chlorophyll was used in the air series, and 2.5 mg. chlorophyll per vessel was used in the nitrogen series. The reaction was run for 30 min.

The similarity in the effects of chloride, *o*-phenanthroline, and CMU, either in air or in nitrogen, on the FMN and vitamin K pathways, under the modified experimental conditions which we now use, has blurred the distinction between the two pathways that was made on the basis of earlier inhibitor experiments [89]. Apart perhaps, from the greater dependence of the FMN pathway on TPN [89] (a dependence that has not yet been reinvestigated under the new experimental conditions), what seems now to distinguish the two anaerobic pathways is the greater requirement, in the case of FMN, for a higher concentration of chloroplast material.

The participation of oxygen in cyclic photophosphorylation may increase the overall rate of ATP formation but only when light is abundant and phosphorylation is limited by a low concentration of cofactors. However, present evidence indicates (Fig. 28) that, in contrast to oxidative phosphorylation, the intervention of molecular oxygen in photosynthetic phosphorylation is an energy-wasteful step that lowers the efficiency of the anaerobic cyclic photophosphorylation process when light is limiting.

On the basis of evidence now available, the participation of oxygen as a catalyst in cyclic photophosphorylation may be represented by the

diagram in Fig. 29. Here the electron flow mechanism (marked by a heavy line) is composed of two parts. The first part is completed when electrons expelled from chlorophyll are accepted by O_2 and, in combination with protons, form water. In the second part, these electrons are replaced by those donated by OH^- , with a concomitant evolution of oxygen, as was described for the non-cyclic electron flow pathway for chloroplasts (Fig. 27). The proposed mechanism, in which oxygen participates, provides for an exchange between molecular oxygen and the oxygen of water and is in agreement with the ^{18}O exchange data recently reported by Nakamoto and Vennesland [163] and Jagendorf [164].



O₂-dependent cyclic photophosphorylation

FIG. 29. Scheme for oxygen-dependent cyclic photophosphorylation in chloroplasts. Details in text.

In summary then, FMN and vitamin K seem to catalyze two pathways of cyclic photophosphorylation, one anaerobic and one catalyzed by molecular oxygen (cf. [62]). The anaerobic pathway, when investigated in an atmosphere of nitrogen, requires appreciable, although still catalytic, concentrations of cofactors and, particularly in the case of FMN, high concentrations of chloroplast material that evidently supply the additional factor(s) needed for the efficient conversion of light energy into ATP under anaerobic conditions. The oxygen-dependent pathway for FMN or vitamin K is catalyzed by very low, "microcatalytic", concentrations of these cofactors and is much less dependent on additional chloroplast material than the anaerobic pathway.

These findings are interpreted to mean that oxygen, when present in a system catalyzed by either FMN or vitamin K, is able to compete effectively with cytochromes for the electrons of cyclic photophosphorylation.

Once the electrons are accepted by oxygen and form water, the cyclic pathway can be maintained only by a release of electrons in the oxygen-forming reaction of non-cyclic photophosphorylation in chloroplasts (Section 13). By contrast, phenazine methosulphate catalyzes the transfer of electrons to cytochrome so effectively [90] that it is able to prevent their "escape" to oxygen and hence the phenazine methosulphate pathway remains an "anaerobic" one even when molecular oxygen is present.

As far as efficiency of conversion of light energy into ATP is concerned, it appears from experiments at limiting light intensities, that the anaerobic cyclic photophosphorylation with FMN or vitamin K is more efficient than with phenazine methosulphate (Figs. 9 and 10). Also, the anaerobic FMN and vitamin K cyclic pathways are more efficient than their oxygen-dependent* counterparts (Fig. 28). These findings suggest the participation of more than one phosphorylation site in the anaerobic FMN and vitamin K pathways (compare Fig. 5 with Figs. 4 and 29).

15. Relation of cyclic to non-cyclic photophosphorylation in chloroplasts

The ability of isolated chloroplasts to carry out both cyclic (Fig. 5) and non-cyclic photophosphorylation (Fig. 27) raises the question of the mutual relation of these two processes. Specifically, what effect would the addition of one of the cofactors of cyclic photophosphorylation have on the reduction of TPN and evolution of oxygen which accompany ATP formation in non-cyclic photophosphorylation?

As shown in Figs. 30 and 31, the addition of either FMN or vitamin K altered non-cyclic photophosphorylation profoundly. ATP formation was sharply increased, whereas oxygen evolution and the accumulation of reduced TPN were abolished. It appears, therefore, that cyclic photophosphorylation is a more "tightly coupled" mechanism for converting light energy into ATP than non-cyclic photophosphorylation. In the

* Distinct from the oxygen-dependent cyclic photophosphorylation discussed here is the "oxidative photosynthetic phosphorylation" [165] by chloroplasts in which oxygen consumption was induced by a *joint* use of a dye (trichlorophenol indophenol), an inhibitor (*o*-phenanthroline or CMU) and DPNH_2 . The correspondence of the term "oxidative photosynthetic phosphorylation" to oxidative phosphorylation by mitochondria appears to be fortuitous. The role of DPNH_2 in this chloroplast system, was not that of a physiological electron donor but that of a non-specific reducing agent for the dye, one of several reducing agents that were effective. That the consumption of oxygen was artificially induced and was only a feature of the special system used, is made clear by the authors' observations that "there was ample energy released by the dark oxidation of the DPNH to form the high energy phosphate bonds. Nevertheless, the reaction gave no phosphorylation unless the system was illuminated, even though the light caused no increase in the rate of oxygen consumption" [165].

presence of the requisite cofactors, cyclic photophosphorylation is capable of diverting all the absorbed light energy for the formation of ATP, and suppressing TPN reduction and O₂ evolution. It is assumed that the intact cell has suitable regulatory mechanisms for keeping cyclic and non-cyclic photophosphorylation in balance.

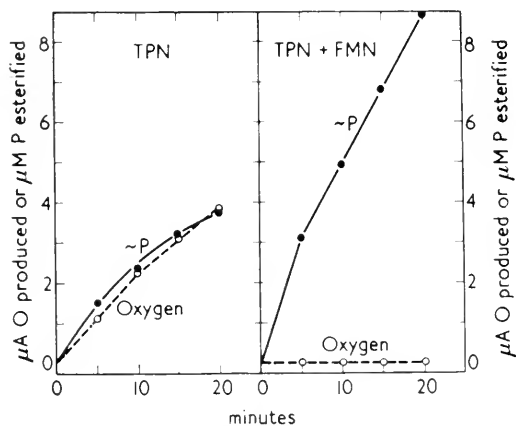


FIG. 30. Photophosphorylation and oxygen evolution by isolated chloroplasts in the presence and absence of FMN (Arnon, Whatley, and Allen, [95, 158]).

TABLE XVII

EFFECT OF FMN AND VITAMIN K₃ ON PHOTOPHOSPHORYLATION AND OXYGEN EVOLUTION LINKED TO TPN REDUCTION

(Arnon, Whatley, and Allen [158])

FMN or vitamin K ₃ added (μmoles)	FMN system		Vitamin K ₃ system	
	P esterified (μmoles)	O ₂ evolved (μatoms)	P esterified (μmoles)	O ₂ evolved (μatoms)
none	5.6	3.6	5.6	3.6
0.0002	6.5	4.2	6.1	3.6
0.0005	7.4	3.8	7.5	2.9
0.001	7.9	3.3	8.0	2.3
0.003	8.4	1.2	9.6	0.9
0.01	9.0	0.4	10.0	0.9

The marked increase in phosphorylation accompanied by a total abolition of oxygen evolution and TPNH₂ accumulation, shown in Figs. 30 and 31, occurred on adding 0.1 μmoles of FMN or 0.2 μmoles of vitamin K (in a final volume of 3 ml.). However, the addition of even extremely minute amounts of either FMN or vitamin K had a measurable effect on

TABLE XVIII

EFFECT OF PHENAZINE METHOSULPHATE (PMS) ON PHOSPHORYLATION AND OXYGEN EVOLUTION LINKED TO TPN REDUCTION

(Arnon, Whatley, and Allen [158])

PMS added (μ moles)	P esterified (μ moles)	O ₂ evolved (μ moles)
none	4.5	4.0
0.003	6.1	4.0
0.01	9.0	3.2
0.1	10.0	0.8

Reaction 4. Table XVII shows that the addition of as little as 0.0002-0.0005 μ moles of FMN or vitamin K increased ATP formation without appreciably depressing oxygen evolution (and the corresponding TPNH₂ accumulation). Similar effects were observed on adding small amounts of phenazine methosulphate (Table XVIII).

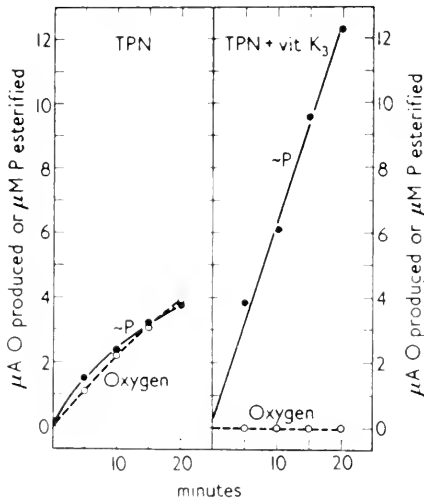


FIG. 31. Photophosphorylation and oxygen evolution by isolated chloroplasts in the presence and absence of vitamin K₃ (Arnon, Whatley, and Allen [95, 158]).

Non-cyclic photophosphorylation provides the three products of the light phase of photosynthesis: O₂, TPNH₂, and ATP. Cyclic photophosphorylation supplies only ATP and the participation of this reaction in CO₂ assimilation would be needed only if the ATP formed in non-cyclic photophosphorylation were insufficient for CO₂ assimilation to the level of carbohydrate. Evidence that this is indeed the case, and that both

cyclic and non-cyclic photophosphorylation are required for CO_2 assimilation, has recently been obtained by Trebst *et al.* [34].

Trebst *et al.* [34] have investigated CO_2 assimilation by isolated chloroplasts in a catalytic system, i.e. one in which, as in an intact cell, TPNH_2 and ATP were present in *catalytic* amounts and CO_2 fixation was therefore possible only in the light while TPNH_2 and ATP were being continuously regenerated at the expense of absorbed light energy. CO_2 assimilation was then investigated, under three conditions: (a) when the

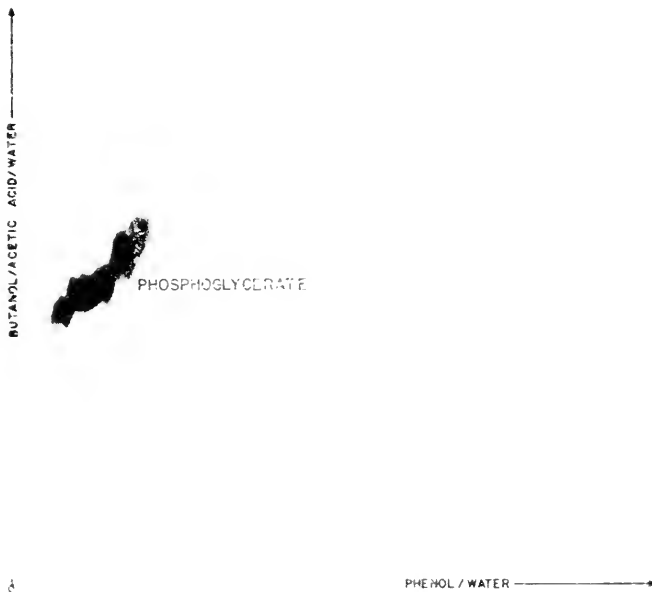


FIG. 32. Radioautograph of a chromatogram showing products of photosynthetic $^{14}\text{CO}_2$ assimilation by illuminated chloroplasts in the absence of added FMN (Trebst, Losada, and Arnon [34]).

photochemical phase was limited to non-cyclic photophosphorylation, (b) when the photochemical phase was limited to cyclic photophosphorylation, and (c) when the photochemical phase included both (a) and (b).

Figures 32 and 33 show that under conditions (a) and (b) CO_2 assimilation was limited almost entirely to the formation of phosphoglycerate. As shown in Fig. 34, the formation of sugar phosphates, which is taken as a measure of a reductive (photosynthetic) CO_2 assimilation in this reconstituted chloroplast system, was observed only in case (c) when a proper balance was established between cyclic and non-cyclic photophosphorylation.

In the experiments illustrated by Figs. 32, 33, and 34, the balance

between the non-cyclic and cyclic photophosphorylation was maintained by adding different amounts of one of the catalysts of cyclic photophosphorylation, FMN, vitamin K or phenazine methosulphate (cf. Tables XVII and XVIII). Concordant results were also obtained by Trebst *et al.* [36] with inhibitor experiments. For example, using the uncoupling effect of ammonia [104, 36] on both cyclic and non-cyclic photophosphorylation, it was possible to suppress the formation of ATP by illuminated chloroplasts without inhibiting the reduction of TPN.

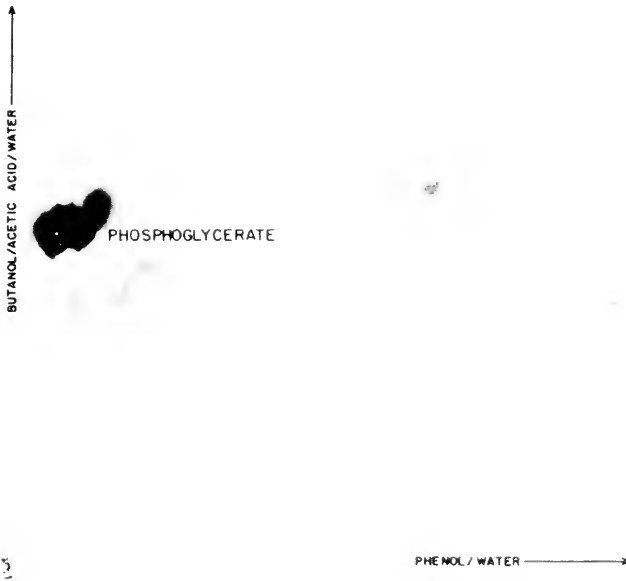


FIG. 33. Radioautograph of a chromatogram showing products of photosynthetic $^{14}\text{CO}_2$ assimilation by illuminated chloroplasts supplied with 0.15 μmoles FMN (Trebst, Losada, and Arnon [34]).

Under these conditions, CO_2 fixation was completely abolished except when the added "acceptor" substance for CO_2 was ribulose diphosphate. In that case a single product, phosphoglyceric acid was formed by the carboxylase reaction which does not depend on added ATP (cf. review [166]). However, no sugar formation occurred because the phosphoglyceric acid could not be reduced by TPNH_2 in the absence of ATP.

Parallel experiments of Losada *et al.* [35] on specific enzyme systems in chloroplasts fortified these lines of evidence and supported the conclusion, that in a reconstituted "catalytic" chloroplast system (in which

CO_2 assimilation can occur only in the light), non-cyclic photophosphorylation alone does not provide sufficient ATP for a reductive assimilation of CO_2 to the level of carbohydrate. Additional ATP must be supplied by cyclic photophosphorylation.

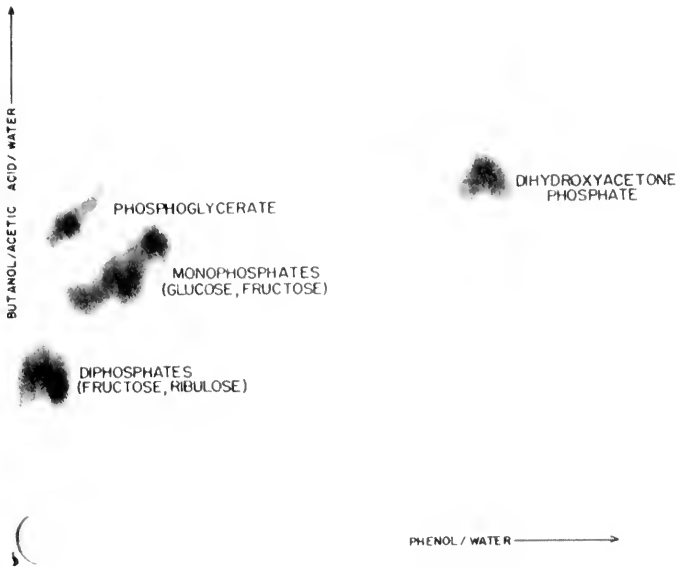


FIG. 34. Radioautograph of a chromatogram showing products of photosynthetic ^{14}C CO_2 assimilation by illuminated chloroplasts supplied with $0.001 \mu\text{moles FMN}$ (Trebst, Losada, and Arnon [34]).

16. The energy conversion concept in photosynthesis

The concept of photosynthesis to which we were led in the 6 years since the process was first completely localized in isolated chloroplasts [13-15] differs from the conventional view of photosynthesis that it is mainly a process of CO_2 assimilation. Photosynthesis appears to be first and foremost a process for converting sunlight into chemical energy and this conversion is more directly associated with phosphorus than with carbon assimilation. In the light of present knowledge, photosynthesis may be defined as the synthesis of cellular substances at the expense of chemical energy formed by photochemical reactions. This definition includes, but is not limited to, CO_2 assimilation.

In both bacterial and plant photosynthesis the photosynthetic events proper are limited to the formation of adenosine triphosphate and reduced

pyridine nucleotide by cyclic and non-cyclic photophosphorylation. This transformation of light into the common currency of cellular energy is fundamentally independent of carbon dioxide assimilation. There is no particular reason why adenosine triphosphate or a photochemically generated reductant could not be used for driving endergonic cellular processes other than CO_2 assimilation.

The photoassimilation of acetate by *Chromatium* is a case of photosynthesis without either oxygen evolution or CO_2 reduction [121]. So is the light-dependent conversion of glucose into starch [128]. All these light-driven reactions are also known to occur in the dark in non-chlorophyllous cells, but in this respect they resemble "photosynthetic" CO_2 assimilation which occurs, by essentially the same pathway, in non-photosynthetic bacteria [32, 33]. Other manifestations of the photosynthetic process, now under active investigation, are the photofixation of nitrogen and the photoproduction of hydrogen gas. Usually, these reactions would be considered as being distinct from photosynthesis proper but according to our present concept these examples represent photosynthetic events because they are being driven by light energy.

In this view of photosynthesis, CO_2 assimilation, although quantitatively the dominant form of photosynthesis on our planet, is fundamentally only a special case of the use and storage of light energy. CO_2 assimilation proper, in both green plants and photosynthetic bacteria, consists of exclusively dark reactions that are not peculiar to photosynthesis.* The familiar accumulation of carbon compounds as carbohydrates during photosynthesis in green plants constitutes storage of trapped light energy. The first products of photosynthesis in green plants [94, 35], ATP and TPNH_2 , are present in the cell only in catalytic amounts and cannot be stored to any appreciable degree for future use, whereas carbohydrates or fats can.

The proposal that ATP formation is a fundamental event in photosynthesis has been made earlier, notably in 1944 by Emerson *et al.* [167], but, as was recently pointed out by Umbreit, "the early experiments were not adequate to demonstrate it" [167]. Without sufficient experimental evidence, the theoretical proposals of Umbreit and his associates could not be adequately defended against the theoretical objections levelled against them (as for example by Rabinowitch [75, p. 229]), particularly since later, the first experiments with ^{32}P to test the occurrence of light-induced phosphorylation in cell-free systems led to negative results.

* A similar conclusion was also reached by investigators of the carbon path in photosynthesis [166, 166a]. Calvin [166a] wrote recently: "The reduction of carbon dioxide, we now have every reason to suppose, occurs in a series of reactions which can take place entirely in the dark. In fact, all the enzyme systems that we now know participate in the conversion of CO_2 to carbohydrates have been found in a wide variety of organisms, many of which are not photosynthetic."

Aronoff and Calvin, who made these experiments with spinach grana, reported that "there is no direct connection between light and the gross formation of organic phosphorus compounds" [168].

17. Photosynthesis and biochemical evolution

The insight into the mechanism of photosynthesis, gained from cell-free experiments with chloroplasts and chromatophores, permits us to interpret, with somewhat enhanced confidence, certain aspects of biochemical evolution which we have already discussed elsewhere [95, 121].

The beginning of photosynthesis may be viewed as an emergence of a porphyrin that gave rise to chlorophyll and permitted the cell to use for metabolic purposes the energy of sunlight. This primitive photosynthesis consisted only of anaerobic cyclic photophosphorylation. No oxygen was evolved and no photochemically formed reductant was required for the photoassimilation of say, acetate, or for the assimilation of CO_2 , as long as hydrogen gas was present in the atmosphere. Oparin [169] and Miller and Urey [170] have summarized the evidence that in the early periods of evolution of life forms, the environment contained hydrogen gas and simple carbon compounds such as acetate. This primitive type of photosynthesis is still seen today in photosynthetic bacteria. *Chromatium*, for example, is capable of using molecular hydrogen for reducing, in the dark, the pyridine nucleotide that is needed for CO_2 assimilation, or of photoassimilating acetate without the aid of an external reductant.

The harnessing of light energy for the synthesis of ATP was an event of supreme importance to the cell. It provided the cell, in an *anaerobic environment*, with a much more efficient mechanism than fermentation for the formation of ATP that was needed for the transformation of existing carbon compounds, into fats, carbohydrates, proteins, etc. Cyclic photophosphorylation gave the *anaerobic* photosynthetic cell a mechanism which, in efficiency of ATP formation, is comparable with the process of oxidative phosphorylation in aerobic cells, that followed it later in the evolutionary scale.

From the point of view of biochemical evolution, one of the most interesting findings in cell-free photosynthesis was that higher, aerobic plants have retained to this day the anaerobic cyclic photophosphorylation as a mechanism for making ATP while sharing with other organisms in the acquisition of the process of oxidative phosphorylation by mitochondria.

As hydrogen gas vanished from the primitive atmosphere, the photosynthetic cell became dependent on an enzymic apparatus for generating photochemically a strong reductant, from such electron donors as succinate or thiosulphate. Light energy now served a dual purpose. It supplied ATP by cyclic photophosphorylation and it provided electrons for reducing

pyridine nucleotides by a non-cyclic electron flow mechanism. In organisms which contain, or can adaptively form, hydrogenase or nitrogenase (photosynthetic bacteria and algae), this phase of photosynthesis can also be observed today as a photoproduction of molecular hydrogen or photo-fixation of nitrogen gas.

The mechanism of anaerobic cyclic photophosphorylation appears to have remained essentially unchanged and constitutes today the common denominator of all photosynthetic cells. The differences between bacterial and plant photosynthesis seem to have arisen from evolutionary transformations of the non-cyclic electron flow mechanism. When it first emerged, the non-cyclic electron flow mechanism was probably of the bacterial type. It could accept electrons from several electron donor

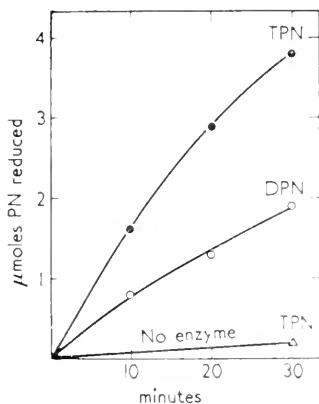


FIG. 35. Pyridine nucleotide reductase from *Chromatium*. Experimental conditions as in Table XIX, except that $K_2H^{32}PO_4$ and ADP were omitted. 4 μ moles DPN or TPN were added as indicated. (Losada, Nozaki, Tagawa, and Arnon [155]; Whatley, Dieterle, and Arnon [161]).

substances (thiosulphate, succinate, etc.) but not from water. Water became an electron donor in the non-cyclic electron flow mechanism only with the emergence of plant photosynthesis.

As was already discussed, the use of water as an electron donor, and the resultant evolution of oxygen, are not essential for the key events in the non-cyclic electron transport, the reduction of TPN and the coupled formation of ATP. When, under special experimental conditions, ascorbate replaced water as an electron donor [160], chloroplasts formed $TPNH_2$ and ATP without the oxidation of water, i.e. without oxygen evolution (Table XIV).

The basic similarity of the non-cyclic electron flow mechanisms in bacteria and chloroplasts is strengthened by the recent isolation by Losada *et al.* [155] of a photosynthetic pyridine nucleotide reductase from

Chromatium and *R. rubrum*. As shown in Fig. 35 the bacterial enzyme catalyzed the photochemical reduction of pyridine nucleotides by chloroplasts that have been deprived of their own pyridine nucleotide reductase. The bacterial PN-reductase was similar to the chloroplast PN-reductase in reducing TPN preferentially to DPN (cf. [86, 148]).

The reduction of TPN was coupled with oxygen evolution when the bacterial enzyme was added to a chloroplast preparation that by itself could not reduce TPN and thereby evolve oxygen (Table XIX). These findings again support the conclusion that TPN reduction and oxygen evolution are basically separate phenomena. The bacterial PN-reductase cannot bring about a coupling of pyridine nucleotide reduction with oxygen evolution in a bacterial system.

TABLE XIX

PHOTOCHEMICAL OXYGEN EVOLUTION CATALYZED BY PYRIDINE NUCLEOTIDE
REDUCTASE FROM *Chromatium*

(Losada, Tagawa, Nozaki, and Arnon [155]; Whatley, Dieterle, and Arnon [161])

Minutes	O ₂ evolved (μ atoms)	TPN reduced (μ moles)
5	0.75	0.76
10	1.60	1.40
20	2.78	2.39
30	3.97	3.18

The reaction mixture contained in a final volume of 3 ml.: washed chloroplast fragments containing 0.3 mg. chlorophyll; and the following in micromoles: tris buffer, pH 7.8, 100; MgCl₂, 5; ADP, 10; K₂H₂PO₄, 10; TPN, 6; and a purified pyridine nucleotide reductase preparation from *Chromatium*. The reaction was run at 15° in the light.

Non-cyclic photophosphorylation enabled green plants to form a CO₂ reductant at the expense of light energy with the aid of an ubiquitous substance, water, and in this way to invade and live autotrophically in areas devoid of reduced sulphur compounds or of other electron donors of restricted distribution. The resultant proliferation of plant growth was responsible for releasing to the atmosphere the oxygen, locked in the water molecule, by the only known important mechanism capable of accomplishing this, photosynthesis of green plants [169, 170].

Once molecular oxygen became available, the way was open for biochemical evolution to progress toward aerobic metabolism. The oxygen-independent cyclic photophosphorylation by chlorophyll-containing particles could now be paralleled by an efficient biological utilization of the energy of chemical substrates through the mechanism of oxidative phos-

phorylation of mitochondria. Photosynthesis of green plants now provided both the substrates and oxygen to make oxidative phosphorylation and aerobic life on this planet possible.

An interesting aspect of the relation between photosynthetic and oxidative phosphorylation in biochemical evolution is the common phylogenetic relationship between proplastids and mitochondria, as it was recently reported by Mühlethaler and Frey-Wyssling [171]. Their observations on proplastid development in embryonic cells suggest that mitochondria followed rather than preceded chloroplasts as functional organelles in cellular metabolism. This is in harmony with the biochemical evidence, since photosynthetic phosphorylation by chlorophyll-containing particles, being independent of molecular oxygen, could occur before oxidative phosphorylation by mitochondria, which requires molecular oxygen (95).

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Discussion

BERGERON: The thiosulphate and light-driven fixation of nitrogen were not cell-free extract studies, is that correct?

ARNON: Yes.

BERGERON: Do you know how far the thiosulphate reaction is from the chromatophores?

ARNON: The thiosulphate reduces the cytochromes of the chromatophores, so it must be close.

ALLFREY: Is there any evidence that polynucleotides are involved here? There is a little RNA and perhaps a little DNA in the chloroplast.

ARNON: There is nothing in our evidence to rule that out.

ALLFREY: Does ribonuclease affect any of these processes?

ARNON: It has not been tried.

DISCHE: What is the relation between your non-cyclic phosphorylation and the phenomenon which Ochoa and Vishniac describe.

ARNON: The Vishniac and Ochoa system involved a collaboration between chloroplasts and mitochondria. Chloroplasts reduced the pyridine nucleotide, which then had to be given to mitochondria to carry out the phosphorylation reaction. Thus the phosphorylation reactions proper were those of oxidative phosphorylation by mitochondria. In photosynthetic phosphorylation no mitochondria are involved; the chloroplasts do it themselves. In the Vishniac and Ochoa phosphorylation by mitochondria oxygen is *consumed*; in non-cyclic photophosphorylation by chloroplasts you will observe that oxygen is *produced*. Moreover, in oxidative phosphorylation by mitochondria, DPNH₂ is oxidized to DPN, in non-cyclic photophosphorylation by chloroplasts it is just the reverse, TPN is reduced to TPNH₂.

DISCHE: I would say that this leads to the question of the source of the hydrogen; didn't Vishniac and Ochoa say that hydrogen came from water?

ARNON: There is no conflict between that statement and the experimental facts of non-cyclic photophosphorylation.

DISCHE: Have you any evidence that phosphorylation and reduction take place in the same process?

ARNON: Yes, that is definite. It has been confirmed in several other laboratories.

DISCHE: But I think that in the mitochondria TPN is not a good phosphorylating agent.

ARNON: DPN is not reduced at all by light in chloroplasts. Chloroplasts are specific for TPN; DPN does not work.

LOOMIS: I would say that one of your slides indicates that oxygen was being released too, because light affecting the chloroplasts would release an oxygen.

ARNON: Quite so. This is a difference between the oxidative phosphorylation by mitochondria in the Vishniac and Ochoa system and non-cyclic photophosphorylation by chloroplasts. In their system oxygen had to be supplied, in our system oxygen is an excreted by-product.

VERNON: This morning you said there was some evidence for photophosphorylation accompanying DPN reduction. Could you expand on this?

ARNON: Yes, we have evidence, which is not yet as extensive as we wish, that non-cyclic electron transfer in photosynthetic bacteria is accompanied by phosphorylation, as would be expected from our postulation; our basic view is that we get a phosphorylation whenever cytochrome is oxidized. When we supply electrons from an exogenous electron donor through cytochromes to chlorophyll and thence to DPN, the cytochromes get oxidized by chlorophyll as the electron transfer occurs. We now have evidence that phosphorylation is also coupled with these reactions in photosynthetic bacteria.

GOLDACRE: In *Nitella*, chloroplasts which are free in the cytoplasm can often be seen rotating at the rate of several rotations a second even in expressed cytoplasm outside the cell. This conceivably can be the result of a flow of current tangential to the surface. We have heard a lot about the movement of electrons, and the evolution of hydrogen and oxygen, and potential differences, and I was wondering if under any conceivable arrangement of the components of chloroplasts you could get current flowing over part of the surface of the chloroplast.

ARNON: I would not wish to speculate so far afield. Suffice it to say that according to our present view what is really important is that we form chemical energy from light. Once the cells form ATP, the cell can use it for different metabolic purposes. With the availability of ATP as a general kind of cellular currency other energy-requiring cellular phenomena are possible and would not necessarily have to be connected with specific electron transfer reactions.

FRENKEL: Warburg in recent publications, has stressed the importance of CO_2 for the Hill chloroplast reaction. I am, therefore, interested in Dr. Arnon's views as he indicated that CO_2 plays no role in the Hill reaction.

ARNON: May I just make this comment. Warburg has proposed that CO_2 reacts *catalytically* in the Hill reaction and that CO_2 cancels out in the overall balance of the reaction. There is nothing in our work which excludes this possibility. What we do maintain, however, is that there is no *net* CO_2 fixation because our experiments were done without added CO_2 and in the presence of KOH.



The Mechanism of the Hill Reaction and Its Relationship to Photophosphorylation*

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Introduction

The purpose of this paper is an exploration of the interrelationship of some of the chemical reactions brought about by the particulate structures derived from the chloroplasts of higher plants. Such green, chlorophyll-bearing particles have been called grana. This term is used here to mean the insoluble fragments obtained when chloroplasts are disrupted in water.

Washed grana have been shown to catalyze two rather different types of reactions which both involve a conversion of light energy into chemical energy. One of these is the well-known Hill reaction [1, 2]; that is, the photoreduction of an added oxidant, accompanied by O_2 evolution. The other is the equally well-known reaction of photophosphorylation, discovered by Arnon and his associates [3], and by Frenkel [4], working in Lipmann's laboratory. In this latter reaction, orthophosphate and ADP are converted to ATP at the expense of light energy. The question I would like to explore is the nature of the relationship between these two rather different phenomena. I would also like to bring up the problem of the relationship between the reactions catalyzed by washed grana and the reactions of photosynthesis in the intact leaf. Let me say at once that I intend to deal only with selected aspects of these problems, and most particularly with some aspects which appear to have been relatively neglected. Let me say also, in advance, that I do not think I have reached any decisive conclusion about the nature of the relationship between photophosphorylation and the Hill reaction. The following account is a progress report to trace the development of our thinking.

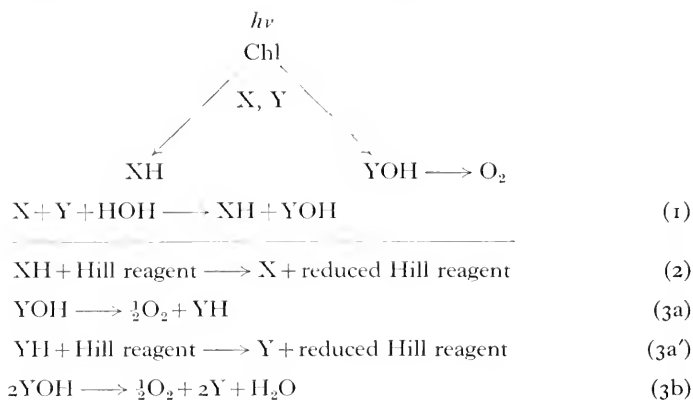
If one wishes to avoid controversy, the safest way of representing the Hill reaction is in a form which is noncommittal about mechanism. Thus, equations 1 and 2 show the reaction with quinone and with ferricyanide

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respectively, and indicate only the chemical identity of the initial reactants and final products.



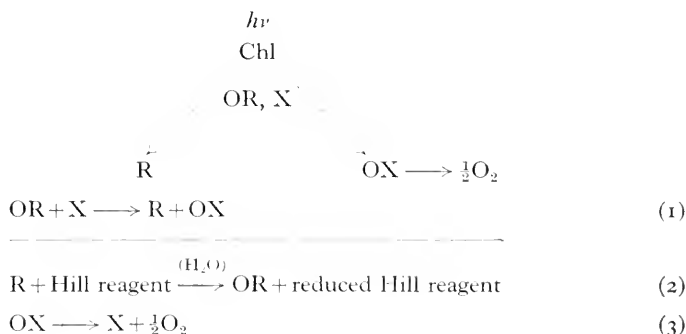
The possible mechanism of the Hill reaction can be represented schematically in many ways [5]. A formulation which has been used quite frequently is depicted in Scheme 1, p. 413. At the top is a diagram often employed to represent the oxidation-reduction reactions underlying the Hill reaction. Photons, in the presence of chlorophyll, supply the energy for a dismutation reaction which results in the formation of a reductant XH, and an oxidant, YOH, from X and Y and water. The reductant is available to reduce an added oxidant, the Hill reagent; and the oxidant somehow excretes its oxygen in the form of O_2 . The photon-requiring step, shown in equation 1, is sometimes referred to as the "splitting of water". X and Y are generally regarded as "built-in"



Scheme 1

components of the chloroplast. Since water is required to balance the equation for the Hill reaction, it is convenient to bring it in at an early stage. We should note, however, that it is impossible to denote how the elements of water participate in the reaction unless we know the structural formulae of all the reaction components. Equations 2 and 3 in Scheme 1 show various steps in the process of reconvertng XH and YOH to X and Y. The regeneration of X is pictured as an oxidation of XH by the Hill oxidant in equation 2. Two possible ways of regenerating Y are shown. One alternative involves the formation of a second reductant, YH (equation 3a), which reduces another molecule of oxidant (equation 3a'). The other alternative shown is a dismutation between two molecules of YOH (equation 3b).

As a reminder of my ignorance of the intermediates of the Hill reaction, I have liked to draw it occasionally as shown in Scheme 2. Here the initial reaction is formulated as an oxygen transfer from OR to X (equation 1) and the regeneration steps are shown in equations 2 and 3.



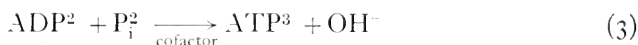
Scheme 2

One might just as well formulate the first reaction as an electron transfer, but the resulting pictures tend to look more complicated. What should be emphasized is that Schemes 1 and 2 are intended to represent more or less the same process, and that we should guard against the danger of reading more information into such schemes than is actually justified by experimental evidence.

Photophosphorylation with a catalytic amount of cofactor

Let us proceed to the nature of the relationship of the process of photophosphorylation to the reactions of Scheme 2.

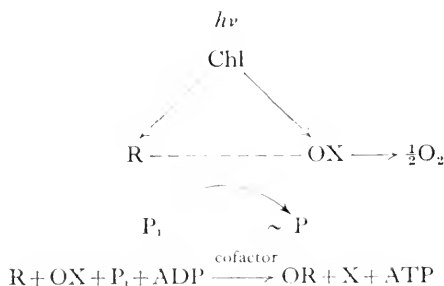
The net process of "cyclic" photophosphorylation is depicted in equation 3.



This was the first type of photophosphorylation recognized [6-8]. It is characterized by the fact that only a catalytic amount of cofactor is added to the chloroplast preparation—the cofactor being any of a large number of oxidation-reduction compounds which must be added (in addition to Mg^{++}), to elicit the photophosphorylation process. It was apparent from the first work of Frenkel that the occurrence of a Hill reaction is certainly not necessary for the occurrence of photophosphorylation. Frenkel worked with bacterial chromatophores which have never been shown to cause a photoevolution of O_2 , but which give an excellent photophosphorylation reaction [4, 9].

There is abundant evidence, however, that chromatophores catalyze

oxidation-reduction dismutations, and that these are associated with the photophosphorylation process [10, 11]. The apparent association of photophosphorylation with electron transport or flow suggested an obvious analogy to mitochondrial oxidative phosphorylation. The simplest way of picturing this analogy is to insert the ATP-generating step into an oxidation-reduction back reaction between the oxidant and reductant generated in the light, as shown in Scheme 3.



Scheme 3

This is the interpretation which was adopted initially and which has been generally accepted [8, 11-14]. It is consistent with the fact that the O_2 evolving step is not a required part of the photophosphorylation reaction. It explains why chromatophores and green grana have this step in common, that is—because they both cause an appropriate oxidation-reduction dismutation—and it explains why the overall Hill reaction may be inhibited without necessarily inhibiting photophosphorylation.

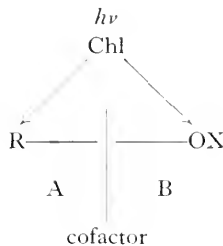
With a mechanism such as that depicted in Scheme 3, the function of the cofactor is to serve as a bridge between the reductant, R, and the oxidant, OX, which are regarded as built-in chloroplast components. The need for such a bridge highlights the separation of R and OX in the chloroplast. Whatever the cause of this separation, whether it be geometry or the absence of an adequate catalyst, R and OX must not back-react freely or the system would not be able to store any chemical energy. The cofactor is pictured as closing a gap and thus permitting electron flow along an electron transport chain from R to OX. The generation of high-energy phosphate bonds is considered to occur in association with the electron flow, in analogy to mitochondrial oxidative phosphorylation.

Though it is a reasonable picture, Scheme 3 cannot be regarded as a statement of known fact. We do not actually know the site of high-energy phosphate bond generation. All that can be said with reasonable certainty is that reaction [3] occurs and that the cofactor is alternately reduced and oxidized while this reaction takes place. It has been suggested more recently [15-17] that the generation of high-energy phosphate bonds may

occur in association with the photon-requiring oxidation-reduction reaction (i.e. equation 1 in Scheme 2). This is a possibility that has to be considered. The mechanism in Scheme 3 has the advantage of providing an analogy to mitochondrial oxidative phosphorylation. The grana contain cytochrome *b* [16, 18] and the quinone derivative Q_{255} [19, 20], and these components are very similar to some of the mitochondrial constituents thought to be rather intimately involved in the oxidative phosphorylation process [21–25]. In mitochondrial oxidative phosphorylation, the phosphate “pick-up” occurs during a dark reaction with a negative free-energy change. This would presumably also be the case in Scheme 3. If the phosphorylation were associated with a reaction occurring against a thermochemical gradient and consequently requiring light energy, one might expect it to be quite different in nature.

Stoichiometric photophosphorylation

If the mechanism shown in Scheme 3 is accepted for the purpose of the argument, a question may be posed with the aid of the diagram in Scheme 4. Here a catalytic amount of cofactor is visualized as shuttling in the manner indicated by the equations. Does the phosphorylation occur during reaction A or B? This question requires an admission that there may actually be several sites for photophosphorylation. Let us assume at this stage that there is only one site.

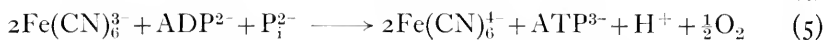
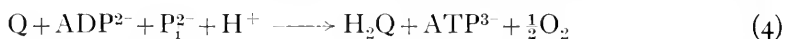


- A. Oxidized cofactor + R \longrightarrow reduced cofactor + OR
 B. Reduced cofactor + OX \longrightarrow oxidized cofactor + X

Scheme 4

There is convincing evidence that if the phosphorylation occurs as depicted in Scheme 3 and at only one site, then this site must be A and not B. It has been shown that photophosphorylation can be coupled to the net photoreduction of Hill reagents such as TPN, ferricyanide, and naphthoquinone sulphate [8, 26–31]. Arnon has termed the process “stoichiometric” photophosphorylation, to distinguish it from “cyclic” photophosphorylation. The stoichiometry of the photophosphorylation

coupled with the reduction of quinone, ferricyanide and TPN is shown in Equation 4, 5, and 6, respectively. These equations have been balanced



completely with respect to hydrogen ions. Note that water is not required to satisfy the stoichiometry (since the oxygen may be regarded as coming from the phosphate), but that hydrogen ions are consumed during the reaction with quinone and formed during the reaction with ferricyanide, whereas there is no net formation or consumption of acid during the reaction with TPN.*

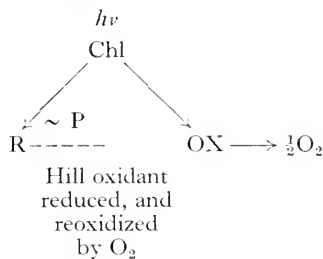
Equations 4-6 show that the $P/2e$ ratio for all three reactions is one. The photoreduction of TPN requires addition of a soluble protein to the grana [32-34], and so may be assumed to be somewhat indirect, but reactions 4 and 5 do not require such a soluble activator. This and other evidence has shown that TPN is not a natural mediator in the reactions with most other Hill reagents [30]. The photoreduction of TPN is discussed in the paper by Dr. Davenport, and so will not be further mentioned here. It is generally agreed that the Hill reactions *per se* can readily be dissociated or uncoupled from the generation of high-energy phosphate bonds. Ageing of almost any preparation of chloroplasts or grana usually (but not always) results in a loss of their capacity for catalyzing photophosphorylation before the loss of their capacity for catalyzing the various Hill reactions. Here again there is an obvious analogy to the behaviour of mitochondria.

The role of oxygen in cyclic photophosphorylation

After the occurrence of stoichiometric photophosphorylation had been demonstrated, a re-examination of the process of cyclic photophosphorylation has led to the conclusion that it generally occurs in a manner somewhat more complicated than that depicted in Scheme 3. If the chloroplasts have an unimpaired oxygen-evolving system, cyclic phosphorylation

* The equations are written for one ionization state of each of the three forms of phosphate (P_1^{2-} , ADP^{2-} , and ATP^{3-}). Since there will be two ionization states of phosphate present at the pH's generally employed for carrying out these reactions, a complete description of the reaction would be more complicated than that indicated. The overall change in acid-base balance indicated by the equation is, however, largely correct. The equations have been balanced in detail to show what is meant by the statement that the chemical nature of X and Y must be known before we can say how the elements of water participate in their oxidation-reduction reactions.

with a catalytic amount of cofactor does not involve reoxidation of the reduced cofactor by OX, but reoxidation by molecular oxygen, as shown in Scheme 5 [31, 33, 35]. Such systems cause a rapid isotopic exchange



Scheme 5

between ¹⁸O₂ and water, and the rate of exchange is as fast or faster than the rate of phosphorylation [36]. The reoxidation of reduced cofactor is partly enzymic and partly non-enzymic. In the latter case H₂O₂ is formed [31]. The proportion of non-enzymic auto-oxidation increases with increasing O₂ tension as evidenced by increased hydrogen peroxide production, without necessarily changing the yield of ATP [35]. This may be taken as additional evidence that the phosphorylation does not occur during the oxidation of reduced cofactor. Warburg *et al.* [31] have described these phenomena for naphthoquinone sulphate as a cofactor, and we have studied them with riboflavin monophosphate [37] and with menadione [35, 38]. In all these cases there is photophosphorylation associated with a Mehler reaction [39].

An exception to the behaviour of the cofactors just described is provided by the N-methylphenazonium salts and the related substance pyocyanine [11, 12, 40-44]. With these compounds as cofactors for photophosphorylation, the predominant, though not the only, mode of cycling appears to be genuinely anaerobic; that is, OX is diverted to oxidize the reduced cofactor. Such phosphorylation is characterized by a low sensitivity to inhibitors of the oxygen-evolving process such as orthophenanthroline, chlorophenyldimethylurea and related compounds, or high concentrations of tris buffer [37, 41, 45, 46]. In the presence of these inhibitors, oxygen becomes strongly inhibitory, as would be expected from the fact that the reduced cofactors are auto-oxidizable, and that if oxygen excretion is not possible the cofactor system must be poised so as to discharge equivalent amounts of R and OX. The oxygen destroys this poisoning. In many respects, chloroplasts with inhibited oxygen evolution show a behaviour toward cofactors reminiscent of that of bacterial chromatophores. The occurrence of the anaerobic cycling confirms the conclusion drawn from the data with chromatophores, that O₂ evolution is not a necessary accompaniment for phosphorylation.

Photoreduction of ferricyanide and of trichlorophenol indophenol

Some of the most interesting results with coupled stoichiometric photophosphorylation have been obtained in a study of the reduction of ferricyanide in the presence of intact chloroplasts by Jagendorf, *et al.* at the McCollum Pratt Institute [26–29]. These investigations started with the original observation of Arnon [8], that the rate of photoreduction of ferricyanide could be increased by addition of ADP and inorganic orthophosphate. The Baltimore group showed that with fresh chloroplasts this increase was a striking phenomenon. The rate of photoreduction of ferricyanide was about 200 μ moles per mg. chlorophyll per hour, in the absence of the phosphorylation system, and about 800 μ moles per mg. chlorophyll per hour in the presence of added orthophosphate and ADP. If the chloroplasts were washed in slightly acid isotonic medium, they were “uncoupled”, in the sense that they caused ferricyanide reduction at a maximum rate in the absence of the phosphorylating system. Uncoupling could also be achieved by addition of ammonia, or of a small amount of the dye, trichlorophenol indophenol. Separate examination of the behaviour of the dye showed that it was an excellent Hill reagent and that its rate of photoreduction was always at least as fast or faster than the rate of photoreduction of ferricyanide under any conditions. The photoreduction of the dye was not associated with coupled phosphorylation, however, and not subject to stimulation by added ADP and orthophosphate to any significant extent.

All the phenomena described up to this point can be accommodated within a simple mechanistic picture involving only one phosphorylation site, designated as A in Scheme 4. The system behaves as though the dye can by-pass the phosphorylation site, reacting more or less directly with R. Such a picture is supported by the results of Witt *et al.* [47], who concluded that the transfer of electrons to indophenol dyes is a simpler and more rapid process than is the transfer to ferricyanide. Their evidence was based on a study of the kinetics of disappearance of a light-induced absorption increase at 515 $m\mu$ in isolated chloroplasts.

“Oxidative” photophosphorylation

The behaviour of trichlorophenol indophenol with chloroplasts exhibits an idiosyncrasy which cannot be so easily explained by the assumption of one photophosphorylation site at A in Scheme 4, unless one makes some fundamental additions to or changes in the Scheme. Although there is no photophosphorylation associated with the reduction of oxidized dye, one can demonstrate a synthesis of ATP from orthophosphate and ADP when the reduced dye is oxidized by molecular oxygen in the presence of

A in Scheme 3, Scheme 6 is drawn to indicate that the dye reacts rather directly with OX as well as with R.

In addition to the first oxidation-reduction dismutation to form R and OX, the Hill reaction with oxidized dye would include reactions A and B. To explain photo-oxidative photophosphorylation we assume also that molecular oxygen can substitute for the other Hill reagents to reoxidize R by way of a phosphorylating electron transport chain. The regeneration of OR and of X should be understood to proceed according to reactions C and D when a sufficient amount of reduced dye is present. The electron transport chain may contain plastoquinone and cytochrome *b*. The cyanide-insensitive auto-oxidizability of cytochrome *b* would be compatible with a position at this point.

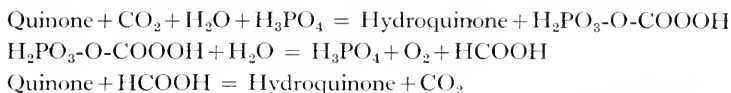
It should be understood in connection with Scheme 6, that though the occurrence of reaction A excludes the occurrence of reaction C for a given molecule of R, and B similarly excludes D, nevertheless, all the reactions could be occurring simultaneously in a given chloroplast suspension, with the relative rates determined by the concentrations of reduced and oxidized dye and by the oxygen tension. "Oxidative" photophosphorylation is rather slow relative to other types of photophosphorylation, although the Hill reaction with the dye is quite rapid. Thus reactions A and B represent the preferred reaction sequence.

There is one very serious difficulty, however, with the otherwise rather plausible picture in Scheme 6. If reactions C and B can both occur, then why should one need a cofactor at all to elicit photophosphorylation or oxygen exchange? If the system is really constituted as shown in Scheme 6, it should form ATP in the light while it evolves and reconsumes O₂, whether a cofactor is added or not. It is established, however, that grana un-supplemented by cofactor do not cause photophosphorylation or oxygen exchange at an appreciable rate.

In order to get around this difficulty one must either postulate different or additional phosphorylation sites, or one must establish a necessity for added cofactor in the oxygen evolving step whereby O₂ and X are formed from OX. Reactions 3a and 3a' of Scheme 1 show in principle how the oxygen evolving step might be dependent on cofactor. Here the evolution of oxygen involves the formation of a second reductant, which is reoxidized by a Hill reagent.

The CO₂ requirement of the Hill reaction

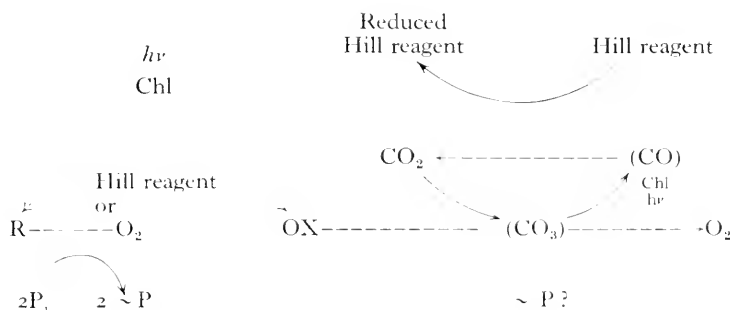
A rather specific mechanism for the Hill reaction recently proposed by Warburg [31] is depicted in Scheme 7.



Net change: $2\text{Quinone} + 2\text{H}_2\text{O} = 2\text{Hydroquinone} + \text{O}_2$

Scheme 7

Warburg's proposal is based in part on the demonstration that CO_2 is an essential requirement for Hill reactions, whether catalyzed by grana or by preparations of *Chlorella* cells [31, 52, 53, 54]. In Warburg's mechanism, the reduction of quinone is pictured as occurring in two separate steps. First one mole of quinone is reduced with the simultaneous formation of a phosphorylated peroxide of carbonate. This peroxide is then converted to O_2 and "nascent" formate, and the latter substance reduces a second molecule of quinone. Scheme 8 shows how the major flaw in Scheme 6 is corrected by the insertion of the elements of Warburg's reaction sequence. What was needed was a requirement for a cofactor in the oxygen evolving step. This need is provided by the requirement of an oxidant for (CO) (i.e. carbon at the oxidation-reduction stage of formate). The continued operation of the catalytic mechanism requires that [CO] must somehow be reoxidized, and we assume that this oxidation cannot be effected by O_2 , even though R can be reoxidized by O_2 , with accompanying phosphorylation. It should be noted that the mechanism shown in Scheme 8 does not



Scheme 8

provide for any retention of reduced carbon, in keeping with the fact that the grana cause no net fixation of CO_2 . The grana are presumably deficient in the means of causing removal of [CO] in a normal manner. It should also be noted that Scheme 8 accounts nicely for the observed facts that the $P/2e$ ratio is about one for photophosphorylation coupled to net reduction of a Hill reagent, whereas the $P/2e$ ratio is about two for phosphorylation coupled to photo-oxidation of reduced dye. The reduced dye is assumed

to be oxidized by OX, with an accompanying reoxidation of R by O₂, over an electron transport chain which gives a coupled phosphorylation of 2 moles of ATP per atom of O reduced. When the Hill reagent is reduced, it replaces O₂ as an oxidant for R, but for each mole of Hill reagent reduced by R, an equal amount is reduced by [CO]. If no net ATP synthesis occurs in the latter reaction, the average P/2e ratio must be one.

Because of the importance of the demonstration that CO₂ is a required component of the Hill reaction, it seemed desirable to verify Warburg's conclusion, particularly for the indophenol dyes which appear to react more immediately with the oxidation-reduction components of the grana than does a Hill reagent such as ferricyanide. In these experiments (which have been done together with Dr. Babette Stern [35, 55]), we wished to measure both the rate of oxygen evolution and the rate of reduction of Hill reagent. Several μ moles of Hill reagent are required in order to obtain reasonably accurate rate measurements of O₂ evolution by the manometric procedures employed in our laboratory. Because of its intense pigmentation, trichlorophenol indophenol could not be employed in these amounts. The reduced dye is rapidly oxidized by ferricyanide, however, in a non-enzymic reaction. We therefore used ferricyanide with a catalytic amount of trichlorophenol indophenol. The procedure involved a determination of the relative rates of photoreduction of the ferricyanide in the presence and absence of CO₂. The CO₂ was removed by the use of KOH in the centre well of the Warburg vessel.

If measurements were made with fresh grana after the usual equilibration period in the dark of about 15 min., little or no difference was noted in reaction rates. It is thus easy to understand why the CO₂ effect on the Hill reaction has often been overlooked. If the dark equilibration was extended over a period of several hours, however, a marked effect of CO₂ developed. The CO₂ appears to be tenaciously held by the preparation, and its removal by KOH in the centre well required a prolonged preincubation in the dark. The longer this preincubation, the greater the CO₂ effect, as measured by the ratio of the photoreduction rate in the presence of CO₂ to the photoreduction rate in its absence.

The above procedures were worked out before the latest papers of Warburg and Krippahl [53, 54] were available to us. It is of interest that the details of procedures we have employed are rather different from those used in the Dahlem laboratory, but that the conclusions are in agreement. We used dye with ferricyanide, saturating light with a small amount of grana, and a long preincubation in the dark. Warburg and Krippahl employed ferricyanide without dye, excess grana, limiting light, and a preincubation period of 1 hr. in the light. They employed a new manometric procedure to show how the rate of photoreduction of ferricyanide varies with CO₂ tension, and they also demonstrated that the effect of CO₂

TABLE I

EFFECT OF CO₂ AND OF TRICHLOROPHENOL INDOPHENOL (TCP) ON THE PHOTOREDUCTION OF FERRICYANIDE

	Ferricyanide reduced (μ moles, mg. chlorophyll/hr.)	
	No CO ₂	1.5% CO ₂
No TCP	73	93
0.03 μ mole TCP	107	240
0.06 μ mole TCP	107	230
0.10 μ mole TCP	88	204

Reaction mixtures contain 100 μ moles of pyrophosphate buffer of pH 6.8, and spinach grana containing 0.2 mg. chlorophyll in a total volume of 3.0 ml. After preincubation in the dark for 2 hrs., 20 μ moles of ferricyanide were tipped in from the side arm and the lights were turned on. $T = 20^\circ$, 4000 ft.-candles white light. Gas phase, either N₂ (with KOH in centre well), or 1.5% CO₂ in N₂.

tension on the Hill reaction rate with grana was the same as the effect of CO₂ tension on the rate of photosynthesis in the intact leaf. Twelve years ago, Boyle [56] reported that CO₂ was required for the photoreduction of quinone. His findings have received little attention, presumably because of his failure to define appropriate experimental conditions for duplicating his results.

The experiment summarized in Table I was one of a series carried out to determine the optimal concentration of dye for demonstration of the

TABLE II

EFFECT OF CO₂ AND PH ON THE HILL REACTION

Initial pH	CO ₂ (1.5%)	Ferricyanide reduced (μ moles mg. chlorophyll hr.)
6.8	-	72
6.7	+	152
6.7	+	173
6.7	-	48
6.6	-	45

The reaction mixtures contained 100 μ moles of sodium pyrophosphate buffer, initially of pH 6.8, 40 μ moles of KCl, 0.07 μ mole of trichlorophenol indophenol, and kohlrabi grana containing 0.2 mg. chlorophyll, with water to make a final volume of 3.0 ml. HCl was added to adjust the pH in the absence of CO₂. The dark preincubation was for 2.5 hr. 20 μ moles of ferricyanide were tipped in from the side arm at the onset of illumination with white light, 4000 ft.-candles. Illumination was for 20 min. $T = 20^\circ$.

CO₂ effect. The results show that the rate of photoreduction of ferricyanide alone, though stimulated by CO₂, is still relatively slow even in the presence of CO₂. When dye is present together with CO₂, however, the rate of photoreduction of ferricyanide is considerably faster than the rate observed when either dye or CO₂ is absent. Since it seems unlikely that CO₂ should be required for the reduction of ferricyanide by reduced dye, the data suggest that it is the photoreduction of the dye itself which is CO₂-dependent. It also seems unlikely that the CO₂ effect is a pH effect, since the addition of a small amount of dye has no appreciable effect on the pH. Table II shows an experiment to verify this conclusion. The small decrease in pH brought about by added CO₂ was duplicated by addition of HCl, with no stimulatory effect on the Hill reaction.

Finally, and most importantly, the CO₂ effect could be shown to be freely reversible [55]. A preparation of grana which has lost activity by prolonged incubation in the presence of KOH is rapidly reactivated if CO₂ is added back a few minutes prior to the photoreduction assay. A representative experiment illustrating the reactivation is shown in Table III.

We regard the above experiments as a partial confirmation of Warburg's results. The requirement of CO₂ for the Hill reaction is another discovery

TABLE III
REVERSIBILITY OF THE CO₂ EFFECT ON THE HILL REACTION

Procedure	Reaction rate in μ moles per mg. chlorophyll per hour	
	Ferricyanide reduced	Pressure increase calculated as O ₂ ($4 \times \mu$ moles O ₂)
1.5% CO ₂ in N ₂ present in dark and light	108	(144)
N ₂ , and no CO ₂ in dark and light	66	62
No CO ₂ in dark, 1.5% CO ₂ in N ₂ added 15 min. before assay in light	101	(142)

The reaction mixtures contained 100 μ moles of sodium pyrophosphate buffer of pH 6.8, 40 μ moles of KCl, 0.07 μ mole of trichlorophenol indophenol, and spinach grana containing 0.2 mg. chlorophyll. Samples were preincubated in the dark for 2.5 hr. Assay in light was for 40 min. Other conditions are those given for Table I. Figures in brackets include CO₂ given off from the bicarbonate of the medium as the result of acid formation attending ferricyanide reduction.

of major significance, which should be listed with the many notable achievements of the Dahlem laboratory. Although our experimental techniques are not as elegant as those employed by Warburg and Krippahl, we feel our results have additional reinforcing value because they were

obtained in a somewhat different way, and they demonstrate clearly the need for CO_2 in the photoreduction of trichlorophenol indophenol, a different Hill reagent from the quinone and ferricyanide employed by Warburg. The demonstrated need for CO_2 in all Hill reactions examined cannot easily be explained in terms of other, previously described CO_2 -fixing reactions, nor can it easily be explained away as an artifact or side effect.

I have already indicated the advantages gained from adding Warburg's mechanism for the Hill reaction to the schemes we have been using to represent the process of photophosphorylation. The diagram shown in Scheme 8 represents our present working hypothesis regarding the manner in which the Hill reaction operates in grana, and the locus of the ATP-generating phosphorylation site in relation to the other reactions. The diagram is subject to amplification and modification. We may speculate that X might include cytochrome *f*. This would be in keeping with Kamen's first postulated chemical step after photon absorption [57]. (See also the paper by Kamen in the present volume.) One might, in fact, borrow Kamen's first step and insert this initial step for the oxidation-reduction between cytochrome and chlorophyll directly into Scheme 8, equating R with reduced chlorophyll, and OX with oxidized cytochrome. Two such reactions would be required, however, for the two electron change shown. Scheme 8 has been drawn to show a second photon-requiring step at the stage where the peroxide of carbonate splits out oxygen. Warburg has not stated explicitly [31] which of the reactions in Scheme 7 require photons, but it seems reasonable to conclude that the second step in Scheme 7 would require light. As Scheme 8 is drawn, the dismutation of OR and X to R and OX would also require light. Two different photon-requiring reactions would be in line with the phenomena of the so-called "second" Emerson effect [57-60], which is possibly related to the activation by blue light described by Warburg [61]. The latter phenomenon appears to be a catalytic effect, however, whereas the Emerson effect does not. In this connection one can speculate about the possibility that the CO_2 "sub-cycle" in Scheme 8 might to some extent operate independently of the generation of R and OX. This does not appear to be impossible.

The relationship of phosphate to the CO_2 "sub-cycle" requires some special comment. Warburg has stated that in addition to CO_2 , the Hill reaction also requires a catalytic amount of phosphate [31]. This is apparently the basis on which he brings orthophosphate into the system to participate in the formation of the precursor of O_2 . In our experiments on the CO_2 stimulation of the Hill reaction we found that added orthophosphate had little effect on the photoreduction reaction rate, but none of our grana preparations was completely free of traces of orthophosphate, so

these findings do not necessarily conflict with Warburg's statement. It is significant, however, that the prolonged incubation at 20° which we employ to remove CO₂ results in a loss of the ability of the grana to give net ATP synthesis in any photophosphorylation system. Furthermore, the ferricyanide-dye system employed does not support ATP synthesis, even with fresh grana. Thus, the operation of the CO₂ sub-cycle shown in Scheme 8 does not appear to require externally added ATP. It is of course possible that high energy phosphate could be transferred more directly, without going through the adenylate system. It seems just as likely that the CO₂ sub-cycle is self-sustaining with regard to high-energy bonds. The oxidation of formate by the Hill reagent involves a sufficient release of free energy to provide for the synthesis of a high-energy phosphate bond, so that no external sources would be required. Scheme 8 was primarily designed to show how Warburg's mechanism for the Hill reaction can be supplemented to account for all the major phenomenology of photophosphorylation, with only one postulated phosphorylation site similar in its chemical components and properties to the site of mitochondrial phosphorylation. As should have been apparent in the development of the argument, the data do not compel one particular choice among a variety of possibilities, so that Scheme 8 should be regarded as a flexible working hypothesis only. The reactions diagrammed in this scheme are all presumed to be catalyzed by washed grana. To explain the overall process of photosynthesis, additional reactions are clearly required. It should be noted that the "formate" of the diagram may be used in part as a source of reducing power, in which event it will be reoxidized to CO₂. Though some of the "formate" will probably also be retained as fixed carbon, the occurrence of this extra CO₂-fixing mechanism does not in any way deny a functional role to the soluble enzymes of the chloroplast which catalyze other CO₂-fixing reactions. It is reasonably self-evident, however, that if the grana photoreduce CO₂ directly in the manner indicated in Scheme 7, then this reaction must be regarded as the most important "CO₂-fixing" reaction in nature. It is conceptually incorrect to think of the grana primarily as generators of ATP and reducing power in the form of reduced TPN. The CO₂ reduction precedes the reduction of TPN instead of following it [54].

The question of a natural cofactor

One final precaution must be kept in mind. The Hill reagents and phosphorylation cofactors used in our studies with grana are largely artificial. This is true even for the FMN- and menadione-stimulated photophosphorylation systems. Although FMN is certainly present in chloroplasts, the quantities are insufficient to elicit any reasonably rapid rate of photophosphorylation. Menadione does not occur in nature, but

the chloroplasts contain a large amount of the chemically related lipid Q_{255} or plastoquinone [19, 20]. This is bound in the grana structure, and Bishop has shown that it is essential for the Hill reaction [19]. We have postulated an associated role in the generation of high-energy phosphate. But it does not play a role equivalent to the cofactor which must be added to elicit the photophosphorylation. Examination of the soluble constituents of leaves has shown, however, that the leaf does contain a substance, or substances, which can function as excellent cofactors for photophosphorylation. My associates, Drs. David Krogmann and Mary Stiller, are currently engaged in a study of this "naturally occurring" cofactor. They find that though this material is present almost exclusively in the supernatant when chloroplasts are centrifuged out of an aqueous medium, there is an appreciable amount of it retained by chloroplasts isolated in non-aqueous medium [62, 63]. Such chloroplasts do not carry out photophosphorylation. The cofactor can be extracted from them and added back to an equivalent amount of chlorophyll in the form of active chloroplasts. Calculated on this basis, there is sufficient cofactor in the chloroplasts isolated in non-aqueous medium to elicit photophosphorylation at at least one-quarter of the maximum rate achieved with FMN or menadione. With larger amounts of natural cofactor, the maximum photophosphorylation rate is as high as the maximum achieved with FMN or menadione. The photophosphorylation with the natural cofactor is oxygen-dependent. In some respects its behaviour suggests that it is an orthohydroquinone derivative. Such substances are widely distributed in leaves. Among them are the flavonoids quercetin and catechin, and related compounds, and the ortho-dihydroxycinnamic acid derivative, caffeic acid, with the related depside, chlorogenic acid. All of these substances, when tested, proved to be good cofactors for photophosphorylation. It is probably in these groups of compounds that we will find a substance or substances which might serve in the leaf to elicit ATP formation. Before we understand completely how the energy of the photons is transmitted chemically to the energy consuming steps in metabolism, we may have to learn a good deal more about the nature of the interaction of the "natural cofactor or cofactors" with the oxidation-reduction components of the grana. In this surmise, as in others, we are following the lead of Warburg, who hinted many years ago at a functional role in photosynthesis for a naturally occurring orthohydroquinone [64].

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Discussion

JAGENDORF: It seems to me that there is a chance that Krogmann's data on the oxygen-requiring phosphorylation with a dye could still be explained if high concentrations of the dye were really an uncoupler and the function of the ascorbate is to maintain the dye in the reduced form so that you have a little bit of the oxidized dye to be an acceptor in the usual scheme making ATP. I think you may be able either to rule this out or prove it one way or the other, by experiments with the indophenol dye and ferricyanide. Were you able to see stoichiometric phosphorylation during the reduction of a mixture of a catalytic amount of the dye and a lot of ferricyanide?

VENNESLAND: We have seen no ATP synthesis with the dye-ferricyanide combination. I should mention that the grana which we were using had been incubated for several hours at 20° in dilute suspensions and would probably not make ATP with any cofactor. When they are fresh they can make ATP with ferricyanide. I do agree with you that there are alternative ways of explaining the phenomena. I think that we must still be very flexible in our views. The mechanism presented is the best working hypothesis at the present time.

SMITH: Is the intermediate compound between CO₂ and formate phosphorylated or is it a peroxyformate?

VENNESLAND: The intermediate which Warburg postulates is a phosphate of a peroxide of carbonate.

SMITH: The question arises as to whether there is a high-energy bond in this intermediate?

VENNESLAND: As Warburg writes it I should say there is a high-energy bond and you would presumably get an energy boost for the liberation of oxygen from this high-energy bond.

LYNEN: We have recently been interested in the interaction of CO_2 with biotin and I should like to ask whether you have tried the effect of avidin on this process?

VENNESLAND: No, we haven't tried it.

DISCHE: In your experiments the rate of the Hill reaction declined with time. Is it not possible that the effect of CO_2 is simply to slow down the decline of the reaction?

VENNESLAND: We could reactivate the inhibited reaction with CO_2 .

LOWENSTEIN: If you want to leave phosphate out of the scheme entirely, then it is easier to envisage oxygen being split out of performic acid than out of carbonic acid.

VENNESLAND: I don't think the evidence that I have available makes it possible to choose between two such alternatives.

LOWENSTEIN: It is hard to visualize oxygen being split out of carbonate as oxygen (O_2).

VENNESLAND: I think Warburg envisages the reaction as occurring on the chlorophyll, with the CO_2 bound in some way. The bound CO_2 could have quite different chemical properties from free CO_2 .

Electron Transport and Phosphorylation in Light-Induced Phosphorylation*

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For some time we have been studying light-induced phosphorylation (photosynthetic phosphorylation, photophosphorylation) in photosynthetic bacteria and also, to some extent, in green plants. In these studies inhibitors of oxidative phosphorylation in animal mitochondria have been employed, in an attempt to obtain information about electron transport and phosphorylation reactions in light-induced phosphorylation, both *per se* and as compared with the system for oxidative phosphorylation.

Washed chromatophores of *Rhodospirillum rubrum* were used in the case of bacteria and washed spinach chloroplasts in the case of green plants. In these two systems light-induced phosphorylation was discovered about 6 years ago, first by Arnon *et al.* [1] in plants and, somewhat later, by Frenkel [2] in bacteria. In fact, most of the present knowledge about the light-induced formation of adenosine triphosphate (ATP)† stems from studies with these materials.

Some results from our earlier investigations, which were made at high light-intensities and under aerobic conditions and which have been described in detail recently [3-7], will be summarized in the first two figures.

Figure 1 shows our proposed scheme for the electron transport in light-induced phosphorylation of *R. rubrum* [3]. The sites of action of certain inhibitors and of the stimulatory agent phenazine methosulphate (PMS) are also indicated. As is seen from the Figure, it is possible in this bacterial system to choose between two pathways for the electron transport. In what may be called "the physiological pathway" it is assumed that the electrons are transferred from the photochemical reductant to flavoprotein and

* This work has partly been carried out in collaboration with Mrs. M. Baltschiffsky (mainly in experiments with bacteria) and Miss B. Arwidsson (mainly in experiments with plants).

† Abbreviations: ATP, adenosine triphosphate; DPNH, reduced diphosphopyridine nucleotide; FAD, flavinadenine dinucleotide; FMN, flavin mononucleotide; PMS, phenazine methosulphate; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; M, moles per litre; LIP, light-induced phosphorylation.

further to a compound X, which represents the site of action for HOQNO [8] and antimycin A [9], and then to the photochemical oxidant. This is a minimum scheme, based on our own results, and there probably exist more electron carriers in the chain. For example, the participation of cytochromes was indicated in earlier work by Smith and M. Baltscheffsky [10]. In what may be called "the PMS-pathway" added PMS, serving as a link between the flavoprotein and the oxidant, gives a new, "artificial"

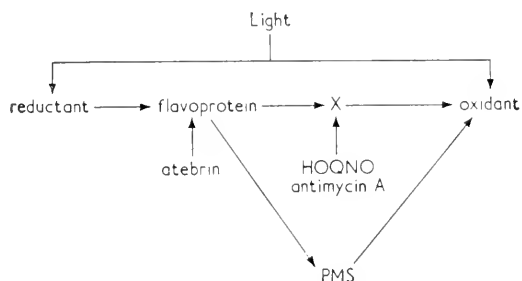


FIG. 1. Electron transport in LIP chromatophores of *R. rubrum*.

electron transport chain. When this pathway is used, the transport of electrons from the reductant to the oxidant along "the physiological pathway" can be eliminated by inhibition at X with HOQNO or antimycin A [9, 11].

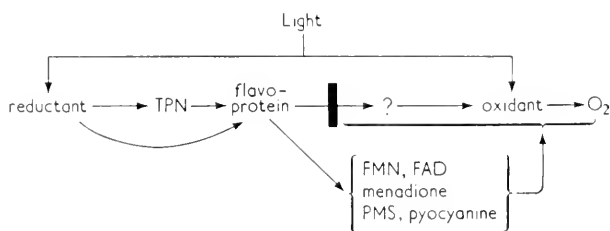


FIG. 2. Electron transport in LIP in spinach chloroplasts.

In green plants, the ATP-formation which is linked to cyclic electron transport is almost totally dependent upon the addition of an electron carrier. Our tentative view about the electron transport in isolated spinach chloroplasts is given in Fig. 2. A great similarity is seen between this scheme and that proposed earlier by Jagendorf [12]. The main difference is that flavoprotein has been included as an obligatory member in the physiological electron transport chain. The experimental background for this scheme has been presented earlier [5].

In recent experiments aiming at an estimation of the efficiency of light-induced phosphorylation *in vitro* we have used three different approaches

to this problem, namely, inhibitor studies, measurements of the quantum requirement of light-induced ATP-formation, and work with pre-aged preparations. It may be pointed out, that all these experiments, except for some inhibitor studies, have been done with bacterial chromatophores, where the above-mentioned possibility of working with two different pathways for the electron transport has been utilized.

Recently, McMurray and Begg [13] reported that an antibiotic named valinomycin completely uncoupled the oxidative phosphorylation in animal mitochondria. The effect of this agent upon light-induced phosphorylation in bacteria and in plants was tested [7, 14].

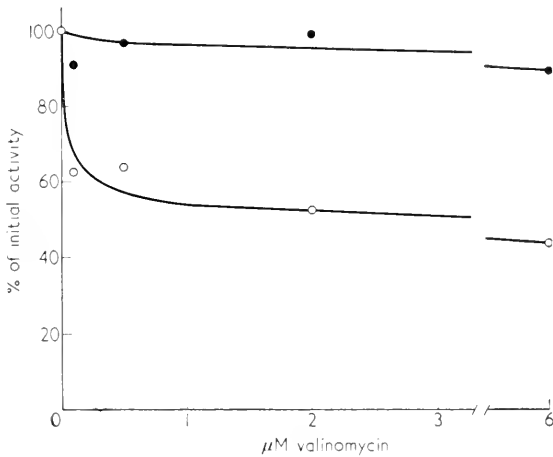


FIG. 3. Effect of valinomycin on LIP in *R. rubrum*. The experimental details were as described in ref. [3]. The final concentrations of the various agents were, where added: 3.3×10^{-4} M ATP, and 3.3×10^{-4} M PMS. ○ = the series without PMS; ● = the series with PMS. Without PMS, 100% activity = 34.5% orthophosphate esterified in 20 min. With PMS, 100% activity = 40.5% orthophosphate esterified in 6 min. In all samples the "OD₈₀₀" was 0.28.

In chromatophores of *R. rubrum* (Fig. 3) less than micromolar concentrations of the antibiotic gave a partial inhibition when "the physiological pathway" for electron transport was used. Titration to much higher concentrations of inhibitor showed a levelling off at about 50% inhibition. On the other hand, phosphorylation in "the PMS-pathway" was not significantly inhibited. Such an absence of effect with low concentrations of inhibitor was encountered also in the experiments with spinach chloroplasts, as is demonstrated in Table I. Irrespective of whether menadione (vitamin K₃), FMN, FAD, or PMS was used as added electron carrier, no marked inhibition was seen with the low concentrations of valinomycin.

The results with valinomycin (Fig. 3) are given the following tentative explanation (Fig. 4). In *R. rubrum*, "the physiological pathway" is connected with *two* phosphorylation sites, one of which is sensitive to valinomycin; thus we have the 50% inhibition. The valinomycin-sensitive site is by-passed when "the PMS-pathway" is used; thus we have no inhibition. In other words, the $P/2e^-$ ratio is assumed to be 2 in "the physiological pathway" and 1 in "the PMS-pathway". According to this, the rate of electron transport in the latter as compared to that in the former is, obviously, twice as high as would be assumed from a direct comparison of the rates of phosphate esterification. The valinomycin-insensitive phosphorylation site, which would be common for both pathways, may well be, in some way, closely linked to the primary photochemical reaction, which has been implied in Fig. 4 by connecting this phosphorylation with the

TABLE I

EFFECT OF VALINOMYCIN ON LIP IN SPINACH CHLOROPLASTS

The experimental details were as described in ref. [5]. The final concentrations of the various agents were, where added: 3.3×10^{-4} M ATP, 10^{-4} M menadione, 1.3×10^{-4} M FMN, 10^{-4} M FAD, and 2×10^{-5} M PMS. In the menadione, FMN, FAD and PMS-series, respectively, the values for 100% activity were 58, 28, 57 and 53% orthophosphate esterified and the chlorophyll content in each sample 0.12, 0.07, 0.12, 0.13 mg. respectively. The time for the experiments was 6 min.

Valinomycin (μ M)	Per cent of initial activity			
	menadione	FMN	FAD	PMS
—	100	100	100	100
0.1	98	113	108	95
1	98	84	96	96
3	90	62	75	114
6	78	40	33	98

photochemical oxidant. Consequently, there may be two kinds of electron transport-linked phosphorylation in the light-induced formation of ATP in bacterial chromatophores, one being similar to and the other different from electron transport phosphorylation in respiring mitochondria, as is visualized from the data obtained with valinomycin.

In spinach chloroplasts (Fig. 4) the absence of inhibition by low concentrations of valinomycin is tentatively taken to indicate the functioning of only one phosphorylation site, corresponding roughly to the valinomycin-insensitive one in bacteria. The possibility cannot be excluded, that ATP-formation at a site of phosphorylation, similar to the valinomycin-sensitive one in bacteria, has been uncoupled in the spinach chloroplasts during the preparation.

By measuring the quantum requirement for the formation of ATP we

have obtained further indications that there may be two sites of phosphorylation in the bacteria, and that one is by-passed in "the PMS-pathway". In these experiments, which were made at the Johnson Research Foundation* in Philadelphia, my wife and I were fortunate to have the collaboration of Dr. John Olson of Brandeis University. Experimental details of this work are published elsewhere [15]. As is seen in Table II, the quantum requirement for the formation of one molecule of ATP was about (although not quite) twice as high in "the PMS-pathway" as in

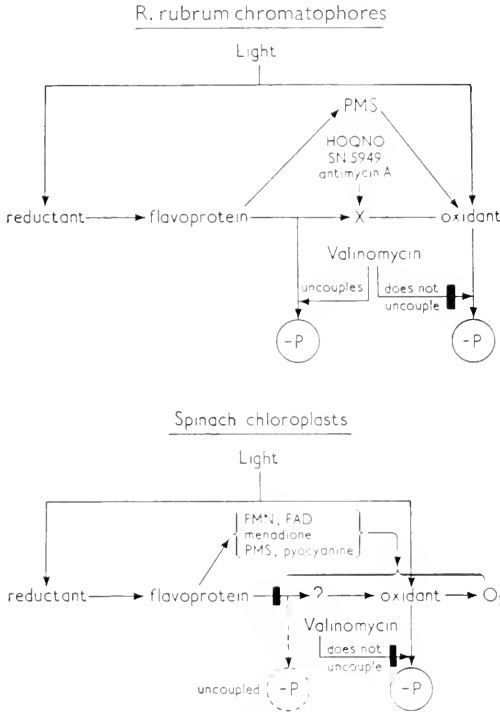


FIG. 4. Light-induced phosphorylation.

"the physiological pathway". This is to be expected if there are twice as many phosphorylation sites in the latter pathway as in the former.

Some of the preparations used in the studies on the quantum requirement for ATP-formation were highly active, giving values of 600-700 μ moles of orthophosphate esterified per hour per mg. chlorophyll (in a volume of 3 ml.). This was obtained in the absence of any added electron carriers. The bacterial extracts were prepared in the manner reported

* The generous support of Dr. Britton Chance, head of the Johnson Research Foundation, is gratefully acknowledged.

TABLE II

QUANTUM REQUIREMENT FOR LIP IN CHROMATOPHORES OF *R. rubrum*

The wavelength of the light was 862 m μ . In experiments I and II sand had been used for the grinding, in experiment III alumina had been used. The values were obtained from the initial slopes of the curves for esterification of orthophosphate versus light-intensity.

Experiment No.	Quanta absorbed per molecule ATP formed	
	No addition	PMS + HOQNO
I	6 \pm 1	10 \pm 2
II	5 \pm 1	8 \pm 1
III	8 \pm 1	> 11

[3], except that the intact cells were disrupted by grinding with sand instead of alumina. The method was developed last year in this laboratory [16].

Pre-ageing the chromatophores at 55-57° influenced light-induced phosphorylation as is shown in Fig. 5. It is the third type of experiment suggesting that phosphorylation may occur at two sites in "the physiological pathway" for the electron transport and at only one site in "the PMS-pathway". As is evident from the figure, pre-ageing decreases the phosphorylation much more in the "physiological" system than in the

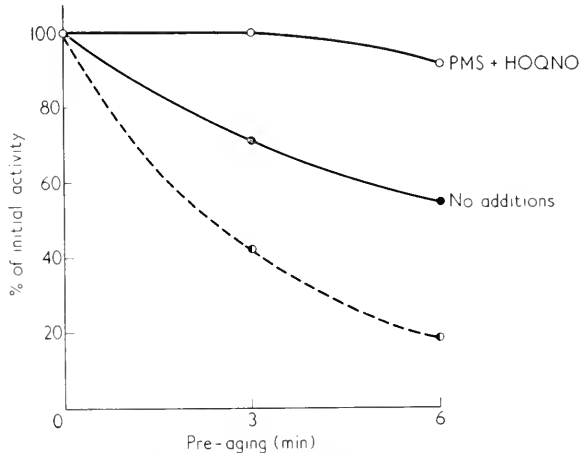


FIG. 5. LIP in *R. rubrum* after pre-ageing. The experiment was performed immediately after the pre-ageing, which was made at 55-57°.

system where PMS and HOQNO have been added. Thus the phosphorylation at the single site of ATP-formation in "the PMS-pathway" appears to be more stable than that at the valinomycin-sensitive site which according to our hypothesis exists in "the physiological pathway"

together with the above-mentioned, more stable site. If the curve in Fig. 5 for "the physiological pathway" represents the decrease of phosphorylation at two sites, one of them being the same as that in the curve indicating a high stability of the phosphorylation in the system containing PMS and HOQNO, the curve for the stability of the other phosphorylation is given by the broken line in Fig. 5.

TABLE III

REQUIREMENT FOR A REDUCING AGENT IN AEROBIC LIP IN
CHROMATOPHORES OF *R. rubrum*

The experimental details were as described in ref. [3]. Twice washed "chromatophores" were used. The final concentrations of the various agents were, where added: $3 \cdot 3 \times 10^{-4}$ M ATP, $3 \cdot 3 \times 10^{-4}$ M succinate, $3 \cdot 3 \times 10^{-4}$ M DPNH, 10^{-2} M ascorbate, $3 \cdot 3 \times 10^{-4}$ M PMS, and 2×10^{-6} M HOQNO. The time for the experiment was 20 min. in the absence and 6 min. in the presence of PMS. The 6-min. values have been recalculated to 20-min. values, assuming earlier shown linearity.

Additions	Per cent orthophosphate esterified
—	0.2
Succinate	9
DPNH	9
Ascorbate	10
PMS + HOQNO	20
Succinate + PMS + HOQNO	22

It was found by Frenkel and by Geller and Lipmann in the early days of research on light-induced phosphorylation [17] that washed chromatophores of *R. rubrum* under aerobic conditions needed catalytic amounts of hydrogen donor, for example DPNH or succinate, in order to produce ATP in the light. Ascorbate may be used instead of these agents [18] as is shown in Table III. High concentrations of ascorbate are needed to give maximum effect (Fig. 6). "The PMS-pathway", however, functions without any addition of hydrogen donor under our standard conditions (Table III). The reason for this can be ascribed to light-induced reduction of PMS, which then provides the necessary reducing equivalents to the phosphorylation system. This explanation has recently been given by Geller and Lipmann [19], who have demonstrated that an added hydrogen donor is indeed needed for phosphorylation when the light-induced reduction of PMS is inhibited by avoiding light of wavelengths where this compound absorbs. Their conclusion was tested with our system and confirmed.

The earlier general attempts to explain the requirement for hydrogen donor [17, 19] may be substituted with a more definite hypothesis on the basis of the effect of ascorbate combined with recent results reported by Chance and Nishimura [20]. Ascorbate has been used to reduce directly mitochondrial cytochrome c in experiments designed to determine the P/O ratio in the span cytochrome c to oxygen [21, 22]. From the known redox-potential of cytochrome c_2 [23] it may be assumed that ascorbate in a similar manner reduces this electron carrier, which has been reported to participate in the electron transport of light-induced phosphorylation in *R. rubrum* [10]. In what appears to be a primary photochemical reaction cytochrome c_2 of *Chromatium* becomes rapidly oxidized in the light, even

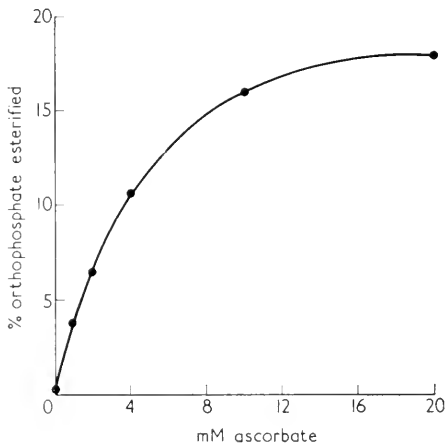


FIG. 6. Effect of ascorbate on LIP in *R. rubrum*. Ascorbate was the only reducing agent added (cf. ref. [3]). The bacteria had been disrupted by grinding with sand.

at 80°K. [20]. The logical assumption has been made that chlorophyll is reduced in this reaction [20]. If the electron transport is initiated by light-induced electron transfer from cytochrome c_2 to chlorophyll it is clear that *some cytochrome c_2 must be present in the reduced form in order for the system to operate*. Aerobically, this criterion may not be fulfilled, and a hydrogen donor which causes either enzymic or chemical reduction of an appropriate amount of cytochrome c_2 has to be present. According to our hypothesis the function of any used hydrogen donor in aerobic light-induced phosphorylation of *R. rubrum* is to reduce an adequate portion of cytochrome c_2 (Fig. 7).

Table IV shows some differences between menadione and PMS as electron carriers in bacterial light-induced phosphorylation [7]. Quantitatively, PMS is a much more potent stimulating agent than menadione,

which gives an appreciable degree of stimulation only in pre-aged preparations [6]. However, also with PMS, in preparations with activities of about 600 μ moles of orthophosphate esterified per hour per mg. chlorophyll, or

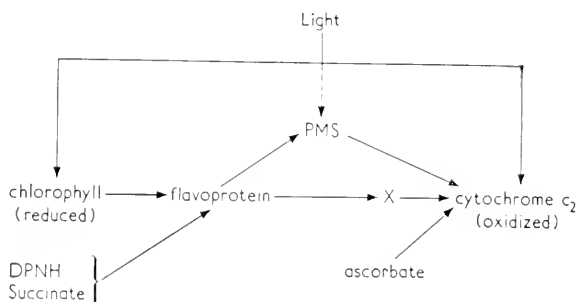


FIG. 7.

higher, the degree of stimulation has been relatively low, as low as 50% or less. The two agents must stimulate phosphate esterification by bridging different parts of the electron transport chain, as antimycin A exerts a

TABLE IV

MENADIONE AND PMS AS ELECTRON CARRIERS IN LIP IN CHROMATOPHORES OF *R. rubrum*

The experimental details were as described in ref. [3]. The final concentrations of the various agents were, where added: 3.3×10^{-4} M ATP, 10^{-4} M menadione, and 3.3×10^{-4} M PMS. The time for the experiment was 20 min. in the absence and 6 min. in the presence of PMS.

Antimycin A μ M	μ moles orthophosphate esterified/h/“OD ₈₀₀ ”					
	I			II*		
	No addition	Menadione	PMS	No addition	Menadione	PMS
—	25	28	91	7	13	74
0.1	1.5	1.1	76	0.4	0.3	66

* In experiment II the chromatophores had been partly destroyed by heating 10 min. in a water-bath of 55-57 immediately before the actual experiment.

strong inhibition only on the stimulation due to addition of menadione. This is seen especially clearly in the experiment with the pre-aged sample. A comparison between the values obtained with the fresh and the pre-aged material further shows that the stability of the three systems increases in the order: system with no added electron carrier, system with added menadione, and system with added PMS. This sequence has been shown

in more detail in other experiments [24]. As another example of the similarities between respiratory electron transport in animal mitochondria and light-induced electron transport in bacterial chromatophores may be mentioned, that also in mitochondria the stimulation of electron transport obtained with menadione is sensitive to antimycin A, as was shown by Conover and Ernster [25], whereas that obtained with PMS is insensitive, as was shown by Kimura and Singer [26].

Earlier it has been strongly emphasized that electron transport in oxidative phosphorylation of animal mitochondria and in light-induced phosphorylation of plant chloroplasts and, especially, bacterial chromatophores shows several similarities [6]. On the basis of data given here this view is further strengthened. For two reasons we consider the chromatophores of *R. rubrum* as being most suitable for studies of the kind presented. The first is that high rates of light-induced phosphorylation are obtained without the addition of any electron carrier. This means that one does not, at least that way, introduce any artificial "by-pass" around a smaller or greater part of the physiological electron transport system. The second reason is that one may select one of two separate cyclic pathways for the electrons to follow, a fact which has opened up new possibilities to gain more knowledge about the reactions involved in bacterial light-induced phosphorylation.

It has been generally assumed, that more than one molecule of ATP may be formed when two electrons are transported through the electron transport chain in light-induced phosphorylation, as has long been known to be the case in oxidative phosphorylation. The three different kinds of evidence given above provide, when taken together, appreciable support for our opinion that two different sites of ATP-formation exist in "physiological" light-induced phosphorylation of chromatophores from *R. rubrum*, i.e. for a $P/2e^-$ ratio of 2.

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Discussion

VERNON: I should like to discuss briefly the effect of ascorbate on photophosphorylation. There is an alternative explanation which also agrees with the earlier experiments of Geller in which he observed an inhibition of photophosphorylation with high concentrations of succinate or with completely reduced phenazine methosulphate. The experiments of Kamen and Newton with *Chromatium* also indicate that if conditions are excessively reducing, photophosphorylation is inhibited. In our laboratory we have found that addition of ascorbate or succinate under anaerobic conditions produces an inhibition of photophosphorylation with chromatophores of *R. rubrum*. This implies that in the presence of air, succinate and ascorbate partly reduce and thus poison the electron transfer agents at an appropriate level, and if an excess of ascorbate or succinate is added the medium becomes over-reducing and photophosphorylation is inhibited. I think the ascorbate data can be better interpreted from the point of view of poisoning of the electron transport agents.

BALTSCHIEFFSKY: Well, the difference between your point of view and our point of view is not one of principle but one of specificity; you say that electron transport agents need to be poisoned and we go one step further and say that cytochrome c_2 is the electron carrier which needs to be poisoned, that is, adequately reduced in order for the system to function under aerobic conditions. Not only ascorbate but also succinate and reduced diphosphopyridine nucleotide act, we feel, by poisoning cytochrome c_2 .

JAGENDORF: I think it is very interesting that both you and Dr. Arnon find a higher quantum efficiency with low light intensities using a system other than phenazine methosulphate. However, I worry about coming to the conclusion that this necessarily means there is one site for phosphorylation when phenazine methosulphate is used and two sites in the other system. I think there are still some alternative interpretations open; for instance, maybe with phenazine methosulphate you waste some quanta because there are two electron transport paths operating, one not phosphorylating at all and the other one involving the same two phosphorylation sites as with FMN, etc. The point is that a direct measurement

of quantum efficiency is not quite the same thing as a direct measurement of the number of phosphorylation sites.

BALTSCHIEFFSKY: I certainly agree that the quantum requirement experiments only imply a ratio of two to one and that they do not give final proof for it. Regarding the possibility that phenazine methosulphate may act at two points, as Dr. Jagendorf suggested; as long as no experiments support such an idea it seems logical to continue to assume that phenazine methosulphate provides only one "by-pass" around "the physiological pathway". The valinomycin data which were presented appear to be inconsistent with a view that the same two phosphorylation sites could be involved in the presence of phenazine methosulphate as in its absence. Taken together with the two other types of evidence given, it seems to us that the quantum requirement experiments motivate the hypothesis that you have two phosphorylation sites in "the physiological pathway" and only one site in "the phenazine methosulphate pathway".

WILLIAMS: I just wanted to ask about valinomycin inhibition: you wrote it on one of your schemes with the inhibited sites towards the reductant but did you have any evidence on this point?

BALTSCHIEFFSKY: No.

WILLIAMS: You just had to put them somewhere?

BALTSCHIEFFSKY: Yes, we had the phenazine methosulphate results and we had to put in the valinomycin-inhibited step somewhere where phenazine methosulphate "by-passes" the "physiological" electron transport chain in order to explain the fact that we did not have an inhibition when this agent was added. The essential point is really this: we have to do here with cyclic electron transport both in the absence and in the presence of phenazine methosulphate. What our data indicate is that one phosphorylation site is in the part of the "physiological pathway", which is shared by "the phenazine methosulphate pathway" and the other site in the part which is "by-passed" by phenazine methosulphate.

ARNON: I would like to associate myself with Dr. Baltscheffsky's interpretation of the phenazine methosulphate data as suggesting that there is probably more than one phosphorylating site and that by using phenazine methosulphate we are by-passing one of them. It seems to me that this is strongly supported by the chemical evidence for the way phenazine methosulphate acts and I rely here on the data of Dr. Massey. I am very impressed by how rapidly phenazine methosulphate reduces cytochrome in his experiments. It is a very effective electron carrier to cytochrome and this, taken together with the evidence from photophosphorylation experiments gives me some confidence that this may be the correct interpretation, namely, that in the presence of phenazine methosulphate electrons go directly to cytochrome and by-pass any phosphorylation sites prior to the cytochromes.

Reduction of Dinitrophenol by Chloroplasts

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Isolated chloroplasts are capable of synthesizing ATP from inorganic phosphate and ADP in light, provided they are supplemented with catalytic amounts of an electron carrier such as FMN, vitamin K₃ or phenazine methosulphate. This ATP formation was named cyclic photophosphorylation by Arnon to distinguish it from the phosphorylation associated with the reduction of substrate amounts of TPN or K₃Fe(CN)₆.

In contrast with the oxidative phosphorylation of mitochondria, which is completely uncoupled by concentrations of DNP (2,4-dinitrophenol) as low as 10^{-5} – 10^{-4} molar, cyclic photophosphorylation of chloroplasts proved to be rather insensitive to DNP. Inhibition was observed only at concentrations at which the Hill reaction was also blocked, that is at about 10^{-3} molar.

In the course of investigations on the effect of DNP on cyclic photophosphorylation it was found that DNP is even capable of catalyzing the generation of ATP by illuminated chloroplasts. As shown in Table I, the optimal concentration of DNP under anaerobic conditions is about 0.6 μ mole per 3 ml. of reaction mixture, which is of the same order of magnitude as the optimal concentrations of vitamin K₃ or FMN.

It has been shown earlier that under anaerobic conditions vitamin K₃ and FMN are involved in separate pathways for cyclic photophosphorylation. The FMN-pathway proved to be more sensitive to the poisons NH₂OH, NaN₃, KCN, and *o*-phenanthroline than the vitamin K₃-pathway. Cyclic photophosphorylation catalyzed by DNP is similar to phosphorylation in the presence of vitamin K₃ as regards the insensitivity to KCN and NaN₃ and the inhibition by dicoumarol, *p*-chloromercuribenzoate, and CMU (3-(4-chlorophenyl)-1,1-dimethylurea). NH₂OH and *o*-phenanthroline, however, are more inhibitory to cyclic photophosphorylation catalyzed by DNP than to phosphorylation catalyzed by vitamin K₃. This difference will be discussed later on.

TABLE I

DINITROPHENOL AS A CATALYST OF CYCLIC PHOTOPHOSPHORYLATION

Additions (μ moles)	μ moles ATP generated during 30-min. illumination
0.5 vitamin K ₃	14.4
0.5 FMN	7.7
3 DNP	2.5
1.5 DNP	7.6
0.6 DNP	10.7
0.3 DNP	7.9
0.15 DNP	2.9
No addition	0.6
0.6 DNP; dark	0.5
0.6 DNP	8.6
0.6 DNP + 30 KCN	7.7
0.6 DNP + 3 NaN ₃	6.9
0.6 DNP + 3 NH ₂ OH	0.9
0.6 DNP + 0.012 CMU	2.9
0.6 DNP + 0.3 dicoumarol	3.5
0.6 DNP + 0.3 <i>o</i> -phenanthroline	1.7
0.6 DNP + 0.3 <i>p</i> -chloromercuribenzoate	2.5

In addition, the reaction mixture included 40 μ moles Na and K phosphate buffer, pH 7.5; 10 μ moles MgCl₂; 125 μ moles glucose; 1 μ mole ADP; 25 K.M. units of hexokinase; 1 ml. of a suspension of chloroplasts in 0.1 M tris (hydroxymethyl) aminomethane buffer pH 7.5, containing 0.5 mg. chlorophyll; and deionized water to give a final volume of 3.0 ml. The reaction was carried out under anaerobic conditions in Warburg manometer vessels as described previously [1].

A number of phenols have been tested for their catalytic activity in the process of cyclic photophosphorylation and the results are given in Table II. It seems that the presence of a nitro group is necessary though not sufficient for the activity of the phenol derivatives.

In order to act as an intermediate electron carrier across some gap in the electron transport chain of isolated chloroplasts, DNP should be transformed by chloroplasts into some reversible oxidation-reduction system. Actually it was found that illuminated chloroplasts are capable of reducing DNP quantitatively to 2-amino-4-nitrophenol, and that the latter compound can serve as a cofactor for cyclic photophosphorylation.

The reduction of DNP is dependent on light and on anaerobic conditions. When the mixture is kept in the dark or when the chloroplasts are illuminated in air, DNP can be recovered nearly quantitatively even after several hours. This is also the case when boiled chloroplasts are illuminated in the presence of DNP. The transformation of DNP into amino-nitrophenol proceeds approximately twice as fast if phosphorylating reagents

TABLE II

CATALYTIC ACTIVITY OF PHENOLS IN CYCLIC PHOTOPHOSPHORYLATION

Active	Inactive
2,4-dinitrophenol	<i>p</i> -nitrophenylphosphate
2,5-dinitrophenol	picric acid
2,6-dinitrophenol	2,4-dinitro-6-methylphenol
2,4-dinitro-5-acetylaminophenol	2,4-dichlorophenol
2-nitro-4,6-dimethylphenol	pentachlorophenol
2-nitro-4,5-dimethylphenol	<i>o</i> -cresol
2-nitroresorcinol	<i>p</i> -cresol
<i>o</i> -nitrophenol	<i>o</i> -methoxyphenol
<i>m</i> -nitrophenol	<i>o</i> -aminophenol
<i>p</i> -nitrophenol (slightly active)	<i>m</i> -aminophenol
2-amino-4-nitrophenol	<i>p</i> -aminophenol
	<i>p</i> -acetylaminophenol
	<i>o</i> -phenolsulphonic acid
	hydroquinone
	catechol

(phosphate, ADP, $MgCl_2$, glucose and hexokinase) are present. The reaction is strongly inhibited by 10^{-4} M *o*-phenanthroline, 4×10^{-6} M CMU, and 10^{-3} M NH_2OH , which are known to inhibit the Hill reaction, and is accompanied by oxygen production (nearly $1.5 \mu\text{mole } O_2 / \mu\text{mole of DNP}$). The inhibition of the photoreduction of DNP by low concentrations of NH_2OH and *o*-phenanthroline may explain the finding that cyclic photophosphorylation catalyzed by DNP is more sensitive to these poisons than is photophosphorylation in the presence of vitamin K_3 , which apparently is independent of the formation of molecular oxygen.

The photoreduction of DNP is strongly stimulated by KCN, but even under these conditions the rate is not higher than about $4 \mu\text{moles DNP reduced/mg. chlorophyll hour}$. DNP reduction thus proceeds at a much lower rate than the reduction of usual Hill oxidants, such as indophenol dyes or ferricyanide. As 3×10^{-4} molar *p*-chloromercuribenzoate shows no inhibitory effect, it seems unlikely that photoreduction of TPN, which is very sensitive to this poison, is involved in the photoreduction of DNP. It is known, on the other hand, that the Hill reaction is resistant to *p*-chloromercuribenzoate.

From these experiments we may conclude that the ability of DNP to catalyze ATP synthesis by illuminated chloroplasts is due to photoreduction of this compound.

Photoreduction of DNP does not occur in the presence of phosphorylating reagents and vitamin K_3 or FMN. This indicates that the conversion of DNP into aminonitrophenol cannot account for the insensitivity of

cyclic photophosphorylation to DNP. As regards the effect of DNP there seems to exist an obvious difference between the mechanisms of photosynthetic and respiratory generation of ATP.

With the exception of *m*-nitrophenol, all nitrophenols which have been found to be capable of catalyzing cyclic photophosphorylation could be converted into reversible oxidation-reduction systems by reduction of the nitro group to the amino group. It was shown, however, that *m*-nitrophenol is not reduced to *m*-aminophenol, but to an intermediate reduction product which may be identical with *m*-hydroxylaminophenol or *m*-nitrosophenol or, more probably, with a conversion product of these compounds. In this connection it is of interest to note that *p*-nitrosophenol was also found to be active as a catalyst of cyclic photophosphorylation. At the moment an effort is being made to elucidate the structure of the photoreduction product of *m*-nitrophenol.

Chloroplasts are also capable of reducing DNP in the dark under anaerobic conditions, but then the presence of FMN and of an excess of TPNH is required. TPNH cannot be replaced by DPNH or ascorbate, nor FMN by vitamin K₃.

Reduction of DNP has not been observed under aerobic conditions, either in the presence or in the absence of KCN. As FMN catalyzes the oxidation of TPNH by chloroplast preparations under aerobic conditions, it seems probable that the dark reduction of DNP is due to the presence of some enzyme which can transfer electrons from TPNH to FMN. This enzyme may be TPNH diaphorase or TPNH—cytochrome *c* reductase, both of which have been shown to be present in chloroplasts by Avron and Jagendorf [2], and Marrè *et al.* [3], respectively. In accordance with this view it was demonstrated that chemically reduced FMN is capable of reducing DNP. When a solution of FMN is illuminated anaerobically by white light in the presence of TPNH or EDTA, the flavin is reduced reversibly, as has been shown recently by Vernon [4]. Subsequent addition of DNP in the dark resulted in reduction of DNP to aminonitrophenol. TPNH was found to be incapable of reducing DNP.

As yet no indication has been found that FMN has some function in the photoreduction of DNP by chloroplasts. The formation of aminonitrophenol in light is affected neither by the addition of FMN, nor by the addition of the flavin antagonists atebtrin and chlorpromazine.

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Discussion

VENNESLAND: I would like to comment on the fact that you find that hydroquinone and catechol are not co-factors for cyclic phosphorylation. We have been working with a variety of diphenolic compounds and we find that if we put in a high enough concentration of hydroquinone or catechol or any compound which has this type of structure, they will support photophosphorylation; we have to put in what appear to be substrate amounts but, nevertheless, we think that the reaction is cyclic. That is, the hydroquinone and the catechol are oxidized mainly non-enzymically—this is why you need such high concentrations—and then the quinones are reduced with accompanying photophosphorylation. Have you looked to see what happens if you go to high concentrations of the substances you tested? Let me add that the reason that we are particularly interested in this is that some of the flavonoids, which are di-*o*-hydroxy compounds, appear to be good cyclic cofactors. These substances occur in leaves, but they have been neglected as possible natural cofactors for photophosphorylation. Perhaps if we are looking for substances that might function in this way physiologically, the flavonoids should be considered.

WESSELS: The compounds which were found to be capable of catalyzing cyclic photophosphorylation showed an optimal activity at a concentration of about 10^{-4} M. For this reason all phenols which we have examined were added in concentrations ranging from 10^{-5} to 10^{-3} M. We have not added substrate amounts of any of the substances compiled in the Table.



The Relationship between "Methaemoglobin Reducing Factor" and "Photosynthetic Pyridine Nucleotide Reductase"

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In his earliest experiments with isolated chloroplasts Hill [1] found that extracts of acetone-dried leaf contain material capable of accepting hydrogen from illuminated chloroplasts with the concomitant evolution of oxygen. Although the pathway of hydrogen transport in this reaction has not been determined, investigations on the leaf extracts led to the first demonstration of the need for a naturally occurring catalyst of reduction in the Hill reaction. Davenport *et al.* [2] showed that neither methaemoglobin nor metmyoglobin was reduced directly by illuminated chloroplasts, but reduction of either was initiated by the addition to the system of an extract of acetone-dried leaf. The methaemoglobin reducing factor (MRF) was found to be associated with a protein fraction in the extracts.

More recently [3] the active protein has been obtained in a state where it is homogeneous both electrophoretically and in the ultracentrifuge. Purification was achieved by electrophoresis on paper after a preliminary fractionation with ammonium sulphate. The product is a protein of small molecular weight (*c.* 19 000), reddish-brown in colour, and it is active in catalyzing the photochemical reduction of a number of haem-protein compounds including cytochromes *b₃* and *c*. The specificity of the catalyst towards illuminated chloroplasts as a source of reducing power, its high catalytic activity and its localization in the chlorophyll-containing cells of higher plants and algae [4] suggested that it plays a part in the transport of hydrogen in photosynthesis. However, the pattern of specificity towards haem-compounds of comparatively oxidizing potential did not appear to be relevant to the energetic requirements of carbon dioxide reduction.

More significant in this connexion was the isolation and partial purification by San Pietro and Lang [5] of a protein factor active in catalyzing the photochemical reduction of pyridine nucleotides. This "photochemical pyridine nucleotide reductase" (PPNR) was prepared by acetone fractionation, isolation of the active material as a protamine sulphate complex with subsequent recovery of the protein from this complex.

In some preliminary attempts to purify further the protein obtained from pea leaves by the method of San Pietro and Lang, using salt precipitation and electrophoresis as additional preparative steps, it emerged that activity towards triphosphopyridine nucleotide (TPN) and metmyoglobin were associated in the same protein fractions at all levels of purification [6]. It appeared therefore that MRF is a highly purified form of PPNR.

Methaemoglobin reducing factor as a catalyst of TPN reduction

The activity of MRF towards TPN at two stages of purification of the protein from pea leaves is shown in Fig. 1. The final electrophoretic step

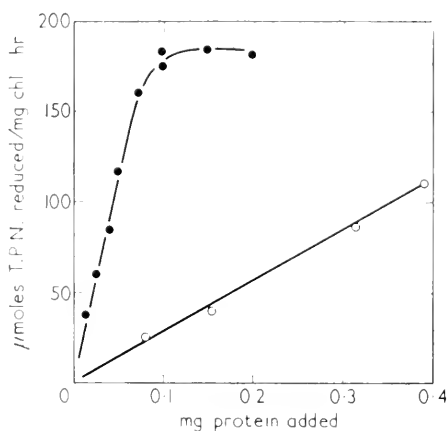


FIG. 1. Catalysis of TPN reduction by "methaemoglobin reducing factor". Pea leaf protein: before electrophoresis; ● after electrophoresis. Reaction mixtures contained (in 3 ml.) added protein as indicated, spinach chloroplasts containing 0.03 mg. chlorophyll, and the following (in μ moles): TPN, 0.4; ADP, 0.5; $MgCl_2$, 20; Na_2HPO_4 , 10; tris HCl buffer, pH 7.7, 150; NaCl, 40. Leaf protein was omitted from the blank cell.

gave a nine-fold increase in specific activity. With the purified protein the particulate chloroplast system was here saturated by the addition of about 100 μ g. to give a rate of reduction of TPN of 184 μ moles/mg. chlorophyll/hr. On the basis of a molecular weight of 19 000 this would correspond to the addition of 5 $m\mu$ moles of the leaf protein.

Comparison of metmyoglobin and TPN as hydrogen acceptors

The relative activity of a PPNR preparation from pea leaves in catalyzing the reduction of metmyoglobin and TPN is shown in Fig. 2. The

protein used here had been further purified by electrophoresis on paper. With either hydrogen acceptor saturation of the chloroplast system occurred at about the same concentration of added protein and at this saturation level TPN was 1.3 times as effective as metmyoglobin in terms of hydrogen equivalents transferred. This ratio, in different experiments, was found to vary from 1.2 to 1.7 but the variation could not be related to the method used in preparing the leaf protein.

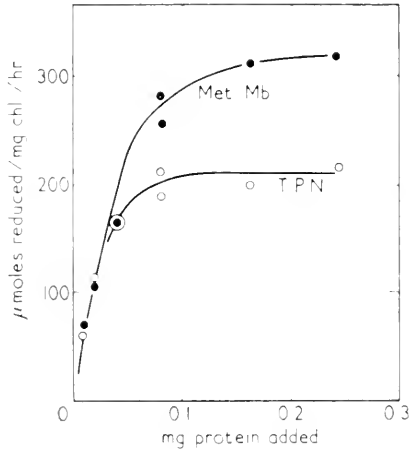


FIG. 2. Comparison of the activity of pea leaf protein (PPNR further purified by electrophoresis) in catalyzing the reduction of TPN and metmyoglobin. Reaction mixture contained (in 3 ml.) leaf protein as indicated, spinach chloroplasts (chlorophyll 0.115 mg.), and (in μ moles) phosphate buffer pH 7.7, 90; NaCl, 40; and the following: TPN, 0.4; ADP, 0.5; $MgCl_2$, 15; ● metmyoglobin, 0.26. Leaf protein was omitted from the blank cells.

Stimulation of TPN reduction by photophosphorylation

The reactions shown in Figs 1 and 2 where TPN was the hydrogen acceptor, were carried out in the presence of adenosine diphosphate (ADP), orthophosphate and magnesium chloride. The presence of this phosphate acceptor system was found to be essential for maximum reduction rates provided that the chloroplasts were at, or near, saturation with respect to added leaf protein. At saturation the additional phosphate-accepting ingredients stimulated the reduction rate 2.5-fold. It was confirmed that inorganic phosphate was incorporated as ATP in the molecular ratio 1 TPNH₂ : 1 ATP [7]. This result is at variance with the report of Jagendorf [8] that no such stimulation of the rate of reduction of TPN during phosphorylation had been detected in three laboratories in the United

States. From the data shown in Fig. 3 it would appear likely that these failures can be attributed to the use of rate-limiting amounts of the catalytic protein. For maximum stimulation all the ingredients of the phosphate-accepting system were found to be required and adenosine 5'-phosphate did not replace ADP unless it was supplemented by catalytic amounts of either ADP or ATP.

In contrast with these observations the rate of reduction of metmyoglobin was found to be unaffected by the presence of the phosphate accepting ingredients and no evidence for photophosphorylation has been

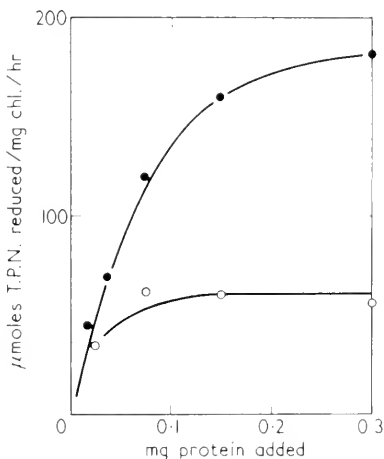


FIG. 3. Effect of phosphate acceptor system on TPN reduction catalyzed by pea leaf protein (PPNR preparation further purified by electrophoresis). Reaction mixtures contained (in 3 ml.) leaf protein as indicated; spinach chloroplasts (chlorophyll 0.016 mg.), and (in μ moles) NaCl, 40; TPN, 0.4; tris HCl buffer, pH 7.0, 150. Reaction mixtures for points ● contained in addition: ADP, 0.5; Na_2HPO_4 , 15; MgCl_2 , 20. Leaf protein was omitted from the blank cells.

obtained with this hydrogen acceptor. Evidence that TPN is able to compete with metmyoglobin for hydrogen produced in the photochemical reaction was obtained by measuring metmyoglobin reduction in the presence and absence of substrate amounts of TPN. The presence of TPN was found to inhibit metmyoglobin reduction and this inhibition was further enhanced when the phosphate accepting system was also present.

At the present state of this work it appears that the haem-protein and TPN reducing activities are common properties of a homogeneous protein. The absence of detectable activity towards diphosphopyridine nucleotide emphasizes a very unusual pattern of specificity towards hydrogen acceptors.

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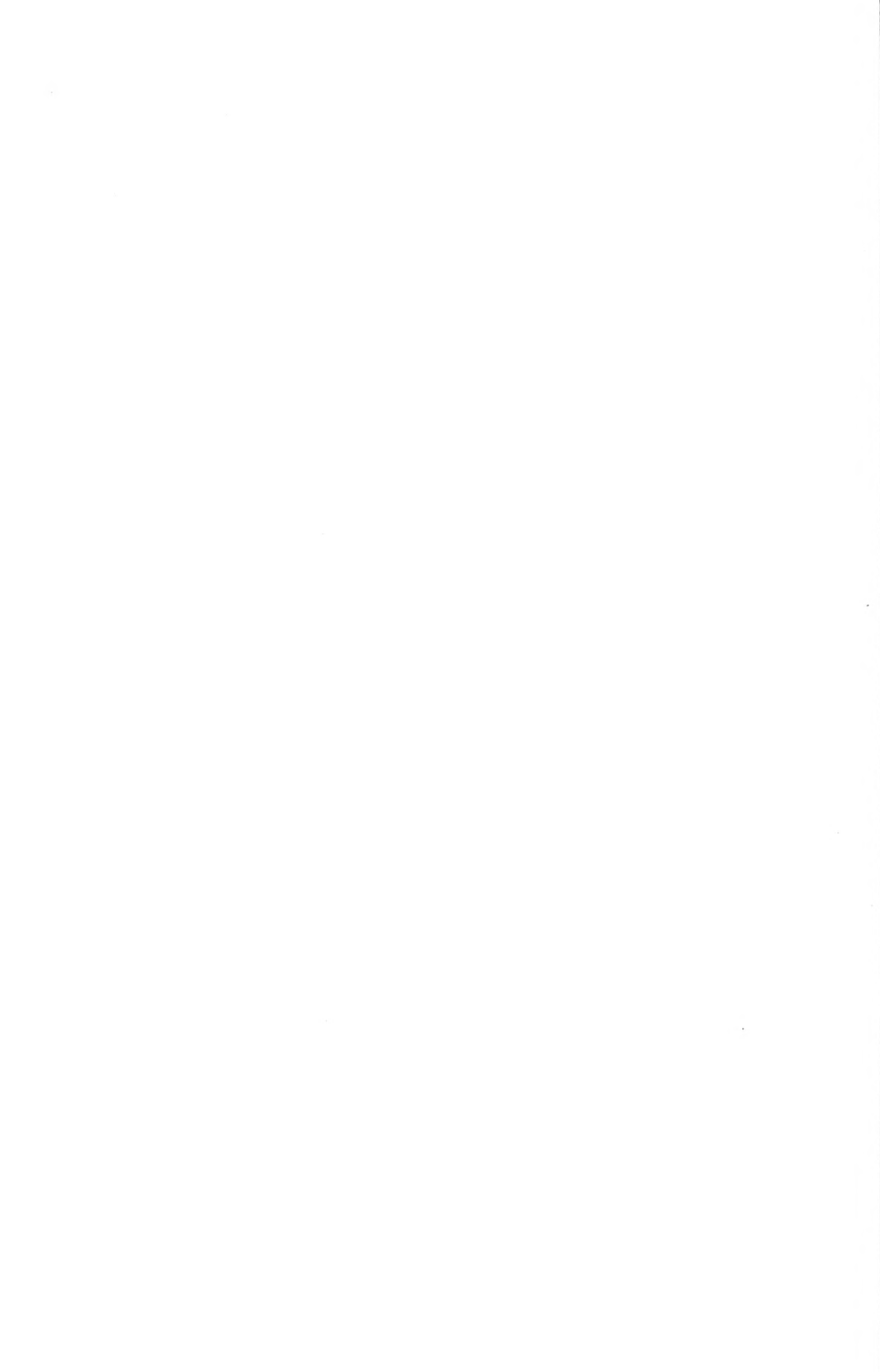
Discussion

JAGENDORF: I should like to mention here that essentially parallel researches are going on at Baltimore, by Dr. San Pietro and associates. If he were here, he could have been given almost the identical paper from his own data. The identities extend to the requirement for higher levels of the reductase enzyme, a three-fold stimulation of the rate of TPN reduction when the complete phosphorylating system is added, and a response to uncouplers.

ARNON: I would like to make one brief comment and to ask one question. I think it is very gratifying that Dr. Davenport and, from what we have just heard, also Dr. San Pietro, find that phosphorylation increases the rate of TPN reduction, because this brings into agreement the facts of non-cyclic photophosphorylation with TPN with the earlier observations of the ferricyanide system. My question is whether you have tested any connection between the pyridine nucleotide reductase and the photosynthetic cytochromes?

DAVENPORT: Well as you know the problem of looking at cytochromes in the presence of chlorophyll is one which, as far as I know, has not been solved unless Dr. Chance can tell us how it can be done, so I cannot say.

CHANCE: Here, I cannot solve the problem of photosynthetic cytochromes but I can mention some very preliminary experiments with Dr. San Pietro who was good enough to work with us on PPNR, and to investigate whether bleaching actually occurs in the presence of the chloroplasts. We did find the pigment in PPNR to be bleached. We found a difference spectrum on illumination of roughly 23 μ moles PPNR and roughly 2.5 μ moles are bleached in the absence of TPN. If TPN is present, bleaching is much less. The rate at which the absorbancy change occurred was in rough agreement with the rate at which TPN was reduced; so it is not unreasonable to believe that this absorbancy change has something to do with the activities. But it is obvious that the spectrum doesn't identify the compound involved.



ATP Formation by Spinach Chloroplasts*†

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The mechanism for conserving oxidation-reduction energy as ATP is as much of a challenge in chloroplasts as it is in mitochondria. It is also a matter of comparative biochemical interest to see how closely these two mechanisms resemble one another.

Our efforts in this area started when it became possible to study the coupling between electron flow and phosphorylation, thanks to the discovery by Arnon and colleagues [1] that ATP is formed during ferricyanide reduction in a Hill reaction. The rate of the Hill reaction is stimulated by simultaneous phosphorylation, up to 3.5 times under the conditions of our experiments [2]. We observed that arsenate was an uncoupler—increasing the rate of electron flow, while inhibiting phosphorylation—but only in the presence of ADP [3]. We have speculated elsewhere [3, 4, 5] that this means *either* that a stable high-energy arsenate intermediate is formed (analogous to the theoretical high-energy phosphate intermediate in ATP formation); *or* that ADP is bound in a high-energy complex first, and phosphate addition is the last step in ATP formation. Since a stable high-energy arsenate intermediate seemed unlikely, we suggested the alternative of a high-energy adenylate as the first step in ATP formation.

The evidence for this sequence, the reverse of that postulated for mitochondria, was indirect. We have since been able to devise a more direct experiment which indicates instead that a high-energy phosphate intermediate is formed first, and ADP addition is the last step. Our later conclusions are in accord with the conclusions to be drawn from recent oxygen-18 studies by Avron and Sharon [6] and by Schultz and Boyer [7].

The procedure for the more direct experiment [8] consists of illuminating chloroplasts in the presence of radioactive phosphate but without ADP. A presumed high-energy phosphate intermediate ($X \sim ^{32}\text{P}$) has a

* Contribution No. 329 from the McCollum-Pratt Institute.

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chance to accumulate under these circumstances. The light is then turned off, stopping electron flow almost instantaneously. A very brief time later ADP is added, allowed to incubate for 15 sec. in the dark, and then the reaction mixture is killed. We find that some [^{32}P]-ATP has formed in the dark, which is therefore a measure of the amount of $X \sim ^{32}\text{P}$ carried in the chloroplasts just after irradiation. The amount comes to 1 μmole per 1000 μmoles of chlorophyll in a remarkably reproducible fashion ($\pm 20\%$).

Although the experiment was simple in concept, scrupulous attention had to be paid to the controls. Figure 1 shows that the chloroplasts were pre-incubated for 8 minutes in the light with a large amount of cold phosphate. This served to convert any internal ADP to unlabelled ATP.

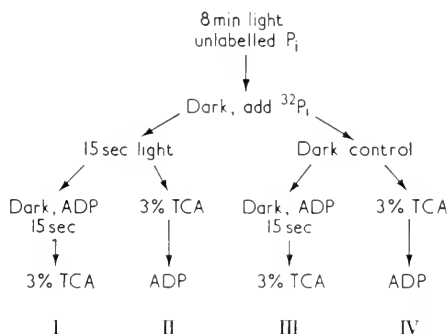


FIG. 1. Protocol for "pre-loading" experiment, designed to show the existence of a high-energy phosphate intermediate.

If this were not done, internal ADP would combine with the added ^{32}P to form large amounts of [^{32}P]-ATP during the second illumination.

Direct controls, as shown in Fig. 1, included adding ^{32}P without a second illumination (treatments III and IV), or killing the reaction prior to addition of ADP in the dark (II and IV).

Table I shows the results of one experiment. Treatment I, representing illumination with ^{32}P followed by addition of ADP in the dark, formed three times as much [^{32}P]-ATP as any of the others. In other experiments this ratio has varied from 1.5 to 6 (but the increment in [^{32}P]-ATP due to illumination remained the same in all experiments). Thanks to the several controls we are sure that [^{32}P]-ATP was not formed in a dark reaction, or in the light prior to the addition of ADP.

By contrast with the successful results of this experiment no [^{32}P]-ATP was ever found as a result of pre-illuminating with ADP and adding ^{32}P in the dark afterwards. We therefore tend to conclude that a high-energy ADP complex is probably not an intermediate here.

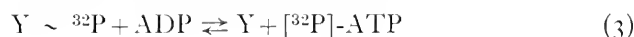
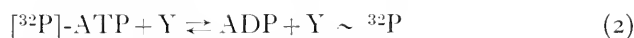
TABLE I

FORMATION OF [³²P]-ATP AFTER ILLUMINATION WITH ³²P

No.	Treatment		[³² P]-ATP (mμmoles/μmole chlorophyll)	Light-Dark
	Illumination	Dark addition		
I	Light	ADP	1.72	1.04
II	Light	TCA	0.60	0.14
III	Dark	ADP	0.68	
IV	Dark	TCA	0.46	

All flasks received 8 min. pre-illumination with cold phosphate. Numbers refer to protocol shown in Fig. 1. The illumination indicated is the second one, of 15 sec. duration, in the presence of ³²P. Reaction mixture contained 0.013 M tris pH 8.0, 0.0033 M MgCl₂, 0.033 M NaCl, 0.00003 M phenazine methosulphate, 0.00033 M phosphate, and chloroplasts containing from 1 to 5 μmoles of chlorophyll; total volume 12 ml.

A trivial possibility in this sort of experiment would be the formation, in the light, of first a very small amount of labelled ATP, and then a larger amount of a compound on a side pathway (such as carbamyl phosphate, or other high-energy phosphate compound). This secondary product might be the storage site for high-energy phosphate, and pass it on to the large amounts of ADP added after illumination. This sequence would be represented by equations 1-3:



If this mechanism were operating the specific activity of phosphate in the secondary product ($\text{Y} \sim {}^{32}\text{P}$), and therefore the total amount of [³²P]-ATP formed in Reaction 3 would be sensitive to variations in the amount of unlabelled ATP, or in specific activity of ATP present before adding ADP in the dark. This is not the case, however.

The internal content of free ATP in chloroplasts is of the order of 200-400 mμmoles/mg. chlorophyll. It can be removed almost completely by breaking the plastids open in water. Whether the internal (unlabelled) ATP is present or absent, 1 mμmole of [³²P]-ATP is formed mg. chlorophyll, owing to pre-illumination followed by ADP in the dark.

A second indication of the absence of a simple dark equilibrium between our stored intermediate and ATP lies in the constant amount of [³²P]ATP formed due to light, over a ten-fold variation in specific activity of internal ATP due to dark reactions. Whether the dark controls have

0.16 or 2.0 μmoles of [^{32}P]-ATP, the increase due to pre-illumination followed by ADP in the dark is 1 $\mu\text{mole}/\mu\text{mole}$ of chlorophyll.

The high-energy phosphate intermediate suggested by these experiments is rather unstable. Our measurements show it has a half-life of about 4 min. in the reaction mixture at 5°.

Isolating and identifying an unstable intermediate may be a formidable job. We have not yet attempted to do so. Instead, we have discovered, solubilized and partially purified an enzyme which might either be, or function close to, our theoretical $\text{X} \sim \text{P}$.

The enzyme is one which causes an exchange of the third phosphate from ATP to ADP. It would appear to be analogous to the ADP-ATP

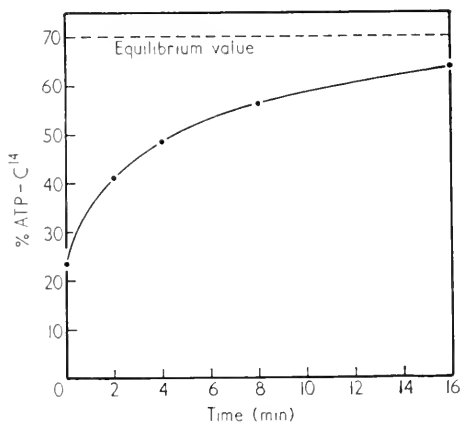


FIG. 2. Time course of ADP-ATP exchange reaction. Reaction mixture was 0.1 ml. total volume, containing [^{14}C]-ADP at 5×10^{-4} M, ATP at 1×10^{-3} M, MgCl_2 at 3×10^{-3} M, tris at 2×10^{-3} M, and solubilized enzyme, the whole brought to pH 8.0. Reactions were run at 30° for the length of time shown.

exchanging enzyme isolated by Wadkins and Lehninger [9]. Activity is measured by incubating labelled [^{14}C]-ADP, unlabelled ATP, the enzyme and Mg for 5 to 10 min. The reaction products are separated chromatographically and counted separately. Figure 2 shows that the label in ATP comes to equilibrium with that in ADP, in proportion to their relative concentrations. The high value in ATP at zero time is due to a 20% contamination of [^{14}C]-ADP (Schwartz Biochem. Co.) with [^{14}C]-ATP.

Purification of the enzyme (to be reported elsewhere) involved extraction from chloroplasts by blending with water, followed by two acetone fractionations and several cycles of freezing and thawing. This results in a solution which has 80% of its protein under one peak in electrophoresis, with two minor components. Upon analysis of various fractions in a preparative electrophoresis apparatus (designed and constructed by

Mr. L. Choules and Dr. R. B. Ballentine at Johns Hopkins [10]) the activity appears to be associated with the major component.

The enzyme characteristics known so far are compatible with a role in phosphorylation. The pH optimum is a broad one, around 7 to 8. Mg ions are needed, with 50% activity at about 5×10^{-4} M. Mg is replaceable by Mn, Fe or Co ions. Although Ca has some low activity by itself, it is inhibitory when combined with Mg. Zn, Hg, and Cu are inhibitors. Ammonium ions are weak inhibitors also, with 50% inhibition occurring at 1×10^{-3} M. Dinitrophenol has virtually no effect.

Efforts to show an irrelevant activity for this enzyme have so far failed. There is no ATP- $^{32}\text{P}_i$ exchange, no myokinase activity, no pyrophosphatase or RNAase, no phosphatase for glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, phosphoglyceric acid or phosphoglycerol. We can rule out glutamine synthetase, protein phosphokinase (either with phosphovitin or casein as substrate) and polynucleotide phosphorylase. No transfer of high-energy phosphate to ADP occurs from carbamyl phosphate, phosphoenolpyruvate, acetylphosphate, phosphoserine or phosphocreatine. Other dinucleotides serve only weakly as phosphate acceptors from ATP: GDP at 9% of the rate of ADP, CDP 5%, UDP 19%, and IDP 14%. No products are formed other than ADP or ATP in the usual reaction, and no other compounds (aside from Mg ions) need to be added even to the highly dialyzed enzyme.

In short, there is no sign of any reaction except for the exchange of a high-energy phosphate from ATP to ADP. There is a very real possibility that the reaction proceeds through a high-energy phosphate on the enzyme:



If this is the case, the E ~ P might be related to or a part of the X ~ P shown in the pre-loading experiments. Our future efforts will be devoted to testing these aspects of the problem.

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Discussion

LEHNINGER: I guess I do not need to say how delighted I am to hear about the occurrence of the ATP-ADP exchange in photosynthetic phosphorylation. This finding of course establishes continuity between oxidative and photosynthetic phosphorylation. The inhibition by ammonium ion is very interesting and I wonder if you have any evidence for a requirement of or an inhibitory effect of either potassium or sodium.

JAGENDORF: No, we haven't tested that. We are suspicious of the significance of ammonium inhibition because it occurs with the highly purified enzyme without any chloroplasts present. The dinitrophenol sensitivity of your ATP-exchanging enzyme requires both the presence of the particles and re-coupling back into them, which is much more suggestive.

LEHNINGER: Well, of course, DNP and ammonium ion do not necessarily uncouple at the same site.

BALTSCHIEFSKY: I have two questions. First, was the light TCA control always higher than the dark TCA control? Second, did you use arsenate instead of phosphate and did you in that case obtain an intermediate; if so, was it more stable or less stable than the phosphate intermediate?

JAGENDORF: The light TCA control will always be a little bit higher than the dark TCA control because in the light when ^{32}P is present, all one needs is a trace of residual ADP, or a little break-down of the large amount of ATP present to ADP, and ^{32}P -ATP will be formed. By being careful in eliminating ADP from the system and especially by using short times to prevent ATP break-down, we can keep this control down to a minimum. Also, if there were a slow exchange reaction you would see it in the control where TCA is added after the light but before ADP; I think that the fact that we have a 15-sec. exposure rather than several minute exposure helps a lot in that respect.

Arsenate does inhibit somewhat if you put it in with the phosphate but whether it forms an intermediate or not I don't know. Even if arsenate did form an intermediate we would not be able to trap it in a stable compound analogous to trapping the high energy phosphate intermediate as ATP.

VENNESLAND: Would you care to comment, Dr. Jagendorf, on the mechanism of the arsenate effect that you observed previously? I am referring to the ATP-dependent stimulation of ferricyanide reduction by arsenate. This appeared to be a real and rather striking phenomenon, and I wonder how you would explain it?

JAGENDORF: Well yes, it is a real phenomenon; at that time we had only thought of two possibilities: (a) that there was a stable high energy arsenate intermediate, or (b) that ADP comes first. Now we find that ADP doesn't come first but another possibility has occurred to us since; perhaps the chloroplasts have membranes, not the external membrane but ones surrounding the actual site of phosphorylation. If penetration of arsenate or phosphate were to require the presence of ADP that would explain the ADP requirement for arsenate uncoupling. However, when we broke chloroplasts up into particles, uncoupling by arsenate still required ADP. This experiment seems to argue against the concept of arsenate entry, which leaves us now with the feeling that there may very well be a stable high-energy arsenate intermediate bound to the enzymes.

SMITH: There is a very interesting effect in photosynthesis which is caused by irradiation of the plant with two wavelengths at the same time which is called the Emerson enhancement. When these two wavelengths are used together they give more photosynthesis than when used separately. It has been found that when these two wavelengths are given alternatively the enhancement occurs as well as given simultaneously and the effect will last as long as 15 sec. between irradiation with the two wavelengths of light. In other words, times can go as high as 15 sec. between the radiation with the long wavelengths and that which is absorbed by chlorophyll *b* or some form of chlorophyll *a*, and my colleagues have been wondering whether it is phosphorylation or something of this nature; or could one have an intermediate of the form that you have suggested? Have you done any action spectrum on this at all to see whether it is formed by chlorophyll *a* or chlorophyll *b*?

JAGENDORF: We previously obtained an action spectrum, and, as you probably know, Hoch and Kok, at Glenn L. Martin Co., Baltimore, have run some action spectra for phosphorylation recently. The picture is still a little bit confused, I think, but briefly it looks to me as if the requirement for the accessory pigment occurs only when oxygen evolution occurs. Now phosphorylation can be supported either by an electron transport cycle, or by the series of reactions leading to oxygen evolution. In the experiments of Hoch and Kok there seems not to have been any oxygen evolution, and, correspondingly, no accessory pigment illumination was needed for phosphorylation. In our experiments I think now that there probably was some oxygen evolution, and we did need accessory pigment illumination for phosphorylation. I want to emphasize that the existing data are not complete and I don't want to sound too positive. But I think quite clearly Kok does find some phosphorylation going when only chlorophyll *a* is being illuminated, which would rule out our present intermediate as the one where the second pigment participates.

PACKER: I would like to comment on the possibility of the existence of an arsenate energy-rich intermediate or sort of factorial approach in our studies of the swelling and shrinking phenomenon in mitochondria. We titrated phosphate to get a certain swelling level and we soon noticed a change in the level of the intermediates, so the interesting thing was that we could do the same type of experiment with arsenate and it titrated to exactly the same swelling level, although the arsenate requirement is slightly different from phosphate and we presume from this that it might indicate that an energy-rich arsenate intermediate could exist.



INTACT CELLULAR STRUCTURE AND
FUNCTION



Chairman's Introduction: Remarks on Control of Structure and Differentiation in Cells and Cell Systems

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I have the honour of opening the section on "Intact Cellular Structure and Function".

It must be my first duty to discuss the significance of the title of our section. It could refer to researches in which only intact cells were used as material. I do not think such a study would be very rewarding. No biologist would refrain from carrying out experiments. The title may rather refer to studies which aim at understanding the structure and function at the level of the intact cell. With such a definition of our task the essential difference between this section and the preceding ones tends to decrease or disappear. I suppose that everybody who studies, for example, control mechanisms in isolated mitochondria or microsomes has the hope that these mechanisms apply also to the living cell. Let us call such systems models. The more complete these are with respect to cellular components the more they may bear upon the conditions in the intact cell. On the other hand we must be aware that when we go from the study of cell components to that of the whole cell we have to count with new interactions that may seem to complicate the situation to a large extent.

A concentric approach by different experimental methods seems to be the strategy to be adopted. Neither of the methods may be able to give a satisfactory answer but an increasing insight may be gained from a combination of the different approaches. Sometimes one may also, on the level of the intact cell, distinguish the alternation between complexity and simplicity to which Dr. Kendrew drew attention when he, on the first day of this Symposium, dealt with the structure of the protein molecule.

For a long time many workers have been interested in the physical state, or let us call it the consistency of the cytoplasm. Before the complicated electron micrographs that Dr. Porter has shown us, this problem seems at first sight rather meaningless. It is also generally agreed that we cannot give an overall estimate of the consistency of the cytoplasm on the

basis of, for example, centrifuge experiments. The consistency may vary in different regions in the cytoplasm as Allen and Allen and Roslansky have confirmed in an elegant way by studies on amoeba [1, 2]. What we study by centrifugation experiments or by other methods may seem in the first place to be the consistency of the ground cytoplasm or matrix in which the other components are embedded. The centrifugation and other methods may thus be able to give us certain information about the changes which occur in the matrix. These changes may indeed be impressive. A stratification of the inclusions occurs very readily when an unfertilized egg of the sea urchin *Arbacia* is submitted to an acceleration of $4000-5000 \times g$ for some minutes, whereas 15 min. after fertilization practically no stratification occurs under the same conditions. These variations in consistency have been particularly studied by Heilbrunn and co-workers [6, 7, 8]. The ground cytoplasm is evidently a very complicated system. It contains a number of elements among them certainly fibrous proteins that may be mainly responsible for the changes in consistency. These changes are often characterized as gellations or solations, which expressions indicate variations in the intermolecular binding forces. We look forward to the time when electron microscopy will be able to demonstrate such changes in the cytoplasmic matrix. As gellations and solations may be localized in definite regions of the cell they may play a role in the cell machinery, particularly in the division of the cell and in differentiation.

Contraction may be regarded as gelation in an accidentally or permanently oriented fibrillar system. Its role in amoeboid movements will be analyzed in Dr. Allen's paper, whereas Dr. Gustafson will demonstrate the great role that apparently random cell movements play in bringing about strictly regulated morphogenetic processes.

In Fig. 1 I have roughly outlined a curve from a paper in press [23]. The unfertilized eggs of the sea urchin *Paracentrotus lividus* were exposed for 15 min. to varying concentrations of crystalline trypsin. After the treatment the eggs were thoroughly washed with pure sea water. Thereafter the eggs were fertilized. When trypsin concentrations of $10^{-5}-10^{-4}$ % were used the pretreatment caused blockage of segmentation in maximally 80-90% of the eggs. The blockage was evidently due to a gelation of the cytoplasm. This was also confirmed by centrifugation. Even before fertilization a decreased stratification was observed in those eggs that had been pretreated by trypsin concentrations in the range of maximum effect on the cleavage. The gelation caused by lower concentrations of trypsin is reversed by higher concentrations of the enzyme. ATP in concentrations of 5×10^{-5} M enhances the gelation effect if it is added after a pretreatment of the eggs with a trypsin concentration that is not sufficient to bring about the maximum gelation of the cytoplasm, cf. first arrow from the left in Fig. 1. Added ATP may in such cases bring the eggs to the maximum

degree of gelation. If ATP is added following exposure of eggs to higher concentrations of trypsin it may enhance the reversal of the gelation.

In Ca^{++} -free solutions of trypsin this latter has a lower gelating effect. Exposure of the eggs to glutathione after pretreatment with trypsin enhances the reversal of gelation. From a number of such experiments it was evident that the gelation is not directly caused by the trypsin treatment but this latter activates an enzyme of the egg cell which has the gelating effect. If the dose (time \times concentration) of trypsin is increased other enzymes are activated which cause a reversal of the gelation. Besides the enzymes of cathepsin B type there are at least three other proteolytic enzymes present in the sea urchin egg with their optimum activity around the neutral point, as was demonstrated by my colleague Dr. G. Lundblad

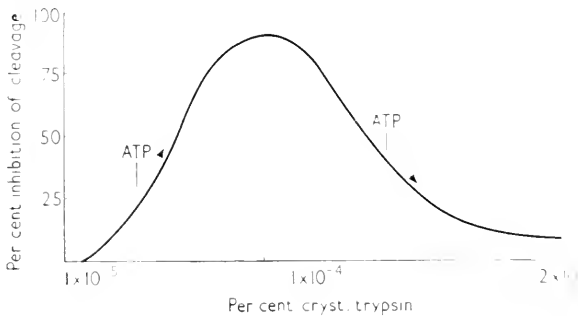


FIG. 1. Simplified curve from Runnström [22], showing the effect of pretreatment of unfertilized eggs with low concentrations of crystalline trypsin. The eggs were fertilized and the number of uncleaved eggs is plotted as a function of the trypsin concentration (duration of pretreatment: 15 min.). The block of cleavage indicates gelation processes in the cytoplasm. If after the trypsin exposure the eggs were transferred to 5×10^{-5} M ATP an enhancement or a removal of gelation was observed according to the level of concentration of trypsin.

[16]. He designates them as E_1 , E_2 and E_3 , of which the first and last are SH-enzymes. It is not excluded that the gelating action may be assigned to one of these enzymes and the reversal of gelation to two other enzymes. These results seem to indicate the possibility that changes in consistency of the cytoplasm may be controlled by enzymes of proteolytic character.

If homogenates of unfertilized eggs were subjected to a treatment with ribonuclease a considerable activation of the proteolytic enzymes occurs. The interpretation was that these enzymes are attached to the ribonucleo-protein granules of the microsome system where they probably have been synthesized.

In conjunction with my colleagues Hagström and Löw [26] I demonstrated that the same holds true for a factor which gels the jelly coat surrounding the egg. This coat consists of a complex of polysaccharides

and protein, cf. [27]. We call the active agent "jelly precipitating factor" which may be identical with the antifertilizin of F. R. Lillie [14]. Later on Hagström found that direct treatment of the surface of intact unfertilized eggs with ribonuclease also causes the precipitation of the jelly coat. Ribonucleoprotein granules are thus probably present also in the surface of the eggs as was earlier suggested by the work of Lansing and Rosenthal [12].

In phase contrast it is easy to see dark spots in the surface layer of the egg, the so-called hyaline layer. The spots represent evidently groups of ribonucleoprotein granules. Upon brief treatment with ribonuclease in sea water they vanish. The hyaline layer is rich in acid mucopolysaccharides. The structureless layer is perforated by numerous villi. These villi are the carriers of the ribonucleoprotein granules which are attached to vesicles or tubules. According to my view the microsome system extends into the tips of the villi. Electron micrographs put at my disposal by my colleague B. Afzelius are in keeping with this view.*

Under certain conditions the villi may be strongly enlarged and in such giant villi both groups of dark granules and of lipoprotein tubules were observed. In certain cases the tubules could be followed as continuous structures deeply into the endoplasm where they were seen to be connected with the astrospheres in the architecture of which tubular lipoprotein structures seem to play a role. One may assume that the microsome system of the villi control the state of the cell surface.

At the segmentation the hyaline layer concentrates to the equator of the egg along with the villi and their content of microsomal elements. A release of a gelating agent occurs equatorially at the onset of segmentation† [24].

Cell differentiation is also on the programme for the discussion today. Dr. W. F. Loomis has discovered how a relatively simple factor, carbon dioxide, may induce the formation of genital cells in the fresh water cnidarian, *Hydra*.

If I may be allowed to persist in talking about our own results, I shall go back to the experiments on the gelating action of trypsin on the sea urchin eggs. Relatively few of the eggs develop that were exposed to 10^{-5} – 10^{-4} %, trypsin. If we examine the larvae obtained after about 24 hr., cf. Fig. 2, we can distinguish three types: (a) rather normal larvae with

* It may be referred to Fig. 22 in [27]. Vesicular and tubular structures are seen in the villi, cf. also Mercer and Wolpert [19]. In the electron micrograph [27] 150 Å granules could readily be seen to surround at least some of the vesicles. The reproduction does not give justice to the original in this respect. What is seen in phase contrast as dark spots corresponds certainly to groups of granules surrounding a vesicle.

† This is also the stage in which the groups of ribonucleoprotein granules are best observed in phase contrast.

endomesoderm formed, (b) larvae with a rather normal ectoderm but the endomesoderm dissolved into large evidently pathological cells, (c) larvae which are animalized, i.e. the whole larva consists only of ectoderm, whereas endomesoderm has not or only incompletely developed. Let us now consider a certain group of cells in the endomesoderm of the normal larva, viz. that marked by a square in Fig. 2(a). From our experiments we must infer that these cells have the potentiality both for endomesodermal and for ectodermal differentiation. In the diagram, Fig. 3(a), the larger horizontal vector (Veg) indicates the pathway of syntheses which have a specifically endomesodermic trend. The opposite horizontal smaller vector (An) represents a pathway for syntheses which have an animal or ectodermic trend. Moreover there are other vectors that represent trends

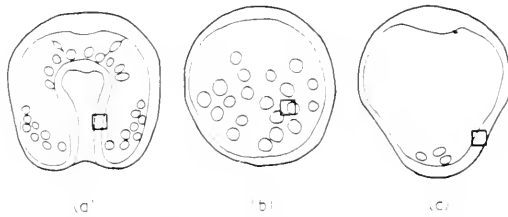


FIG. 2. (a), (b), (c), three possible alternatives of differentiation in eggs that had been pretreated in the unfertilized stage with $6 \cdot 5 \times 10^{-5}\%$ trypsin. In (a) development is normal, a certain region marked by a square becomes normal endoderm. In (b) the ectoderm forms a continuous layer that presents a lower degree of differentiation than (a). The endomesoderm is dissociated in rounded cells. The region corresponding to the marked region in (a) consists of dissociated cells showing tendency for cytolysis. In (c) ectodermization or animalization has occurred. The marked cells again constitute an epithelium but this has ectodermic character. Some few mesenchyme cells appeared.

that are common to cells on all levels in the larva. The vegetal pathway dominates in this region but the animal pathway is not suppressed altogether. It contributes to the character of the cells on this level. In this way one explains the fine gradation in the properties and behaviour of the cells which Dr. Gustafson has demonstrated and certainly will refer to later today. In larvae of the type of Fig. 2(a) no injury was observed, whereas in a larva of the type Fig. 2(b) the threshold of injury evidently was low. In the larval region marked by the square protein synthesis gradually became blocked, with ensuing dissociation of the cells gradually followed by cytolysis. The break up of the synthetic pathways is also indicated diagrammatically in Fig. 3(b). The larvae of type Fig. 2(c) demonstrate however, that the vegetal pathway evidently has a lower threshold than the animal one. The vegetal pathway may now be eliminated or reduced. In this way animalization results, cf. also diagram, Fig. 3(c). We find that

the marked region now consists of cells that are typical for a certain region of the ectoderm. The cause of the injury was in the cases Fig. 2(b) and 2(c) the pretreatment with a low concentration of trypsin. This has induced the activation of certain proteolytic enzymes probably of proteolytic character in the egg. If the activation is limited or reversed normal development occurs (Fig. 2(a)). If this does not occur the activation continues and the proteolytic activity is such that the synthetic pathways may be interrupted. It was concluded previously that the vegetal pathway

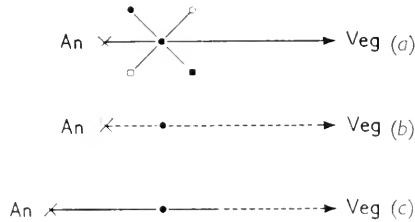


FIG. 3. Diagram to illustrate the condition with respect to the vegetal and animal vectors or pathways at the level of the square in the larvae represented in Fig. 2. In (a) the vegetal pathways of synthesis dominate (Veg), but certain pathways of animal synthetic processes are also open. The four shorter lines represent pathways that are common to all levels in the embryo. In (b) both the vegetal and the animal pathways are interrupted. In (c) the vegetal pathways have been reduced with a compensatory increase in the animal pathways which bring about an animalization of the marked region.

is more easily blocked than the animal one which is the cause of animalization shown in Fig. 2(c). The animal or animalized cells are in general more resistant to injury than the vegetal ones. The vegetal pathway gives the impression of being more unstable.*

* The recent results of Leone [13] are well in keeping with the views presented above. He carried out a set of experiments on the effects of ribonuclease on embryos of the sea urchin *Arbacia lixula*. He found that the enzyme tends to inhibit development in general and especially the differentiation of endoderm, without animalizing the embryos. His results may be explained on the basis of our diagram Fig. 3 (b). On a vegetal level both the animal and vegetal pathways are interrupted by the treatment with the ribonuclease because the formation of ribonucleic acid is the prerequisite for protein synthesis along both pathways. As a consequence also the prerequisite for animalization of the vegetal region is lacking. The somewhat greater resistance of the animal region of the embryo found by Leone corresponds to the result illustrated by our Fig. 2(b). The latter case may correspond to a stronger activation of hydrolytic enzymes in the vegetal as compared with the animal region. On the other hand structural differences may play a role in making the sensitive sites of the macromolecules in question more or less accessible to the attack of the enzymes. The vegetal part of the embryo is also more sensitive to disturbances in electrolyte composition of the medium, as to lack of K^+ or SO_4^{2-} , cf. [21].

The vectors of Fig. 3 may primarily represent pathways of protein synthesis. As experiments with labelled precursors show, incorporation of for example [^{14}C]-leucine into proteins runs parallel with the incorporation of [^{14}C]-adenine into ribonucleic acid, cf. Markman [18]. It is evident that the ribonucleic acid plays the same fundamental role in protein synthesis in the sea urchin material as elsewhere. As far as it is possible to resolve the sites of incorporation, that of the ribonucleic acid precursor (e.g. [^{14}C]-adenine) occurs primarily in the nuclei. It seems thus probable that the synthesis of ribonucleic acid on which the protein synthesis is dependent occurs in the nucleus. I trust that the question about the site of ribonucleic acid formation will be more deeply discussed in the paper by Dr. Prescott who refers to another material, viz. amoebae.

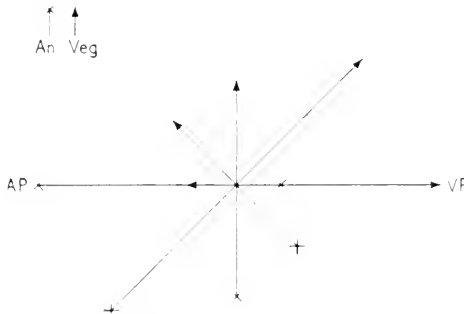


FIG. 4. Diagram of the vegetal and animal vectors over the whole embryo, VP vegetal pole, AP animal pole. Vectors representing the opposite synthetic pathways on the same embryonic level form the same angle with the vegetal-animal axis VP-AP.

In this context anabolic processes other than ribonucleic acid and protein synthesis must be disregarded. It may only be noticed in passing that my colleague Immers found also indications of a parallelism between incorporation of amino acids into proteins and an incorporation of [^{35}S]-sulphate into mucopolysaccharides.

Figure 4 gives a diagram of the vegetal and the animal vectors of ribonucleic acid and protein synthesis over the whole embryo from the vegetal (VP) to the animal pole (AP). The levels in the embryo are given by the angles that the vectors form with the baseline (AP-VP). There is no need to emphasize how grossly simplified this picture is. Nevertheless it may be of some use. Different kinds of ribonucleic acids must be formed in the nuclei where they presumably receive their "information" from the deoxyribonucleic acid, cf. discussion in [3].

As indicated in Fig. 3(a), certain pathways of synthesis may be independent of the level in the animal vegetal system. In Fig. 4 emphasis is

laid, however, on the pathways that are responsible for the animal and vegetal trends of differentiation. The scheme will indicate that the differences between the different embryonic levels are primarily of a quantitative rather than of a qualitative nature. One of our simplifications is certainly to refer to animal or vegetal pathways instead of to families of animal and vegetal pathways. It is to assume that each single pathway corresponds to the formation of one specific ribonucleic acid. The intensity of its formation is, however, regulated in the system. According to our view the primary control is exerted by an animal and a vegetal cytoplasmic centre. Each of these produce certain agents which spread in the direction of the opposite pole, cf. [21, 25]. This view is well supported by a great number of experiments, involving operative separations and transplantations, or transformations obtained by chemical means as those described above, cf. [9, 15, 21].

The main point of attack of the controlling agents is probably the nucleus and particularly the synthesis of ribonucleic acid within the nucleus [18, 25].

So far chemical changes of the ribonucleic acids have not been directly demonstrated during the development of the sea urchin embryo, cf. [5]. It is of some interest that by certain cytochemical tests differences between the nuclear ribonucleic acids of the animal and those of the vegetal embryonic region could be demonstrated. The tendency for "unmasking" of the phosphate groups of the ribonucleic acid seems to be greater in the former than in the latter, cf. [10, 17] and a recent review [25]. These differences become obvious only in the stage in which the primary mesenchyme begins to immigrate. The gastrulation initiates more direct interactions between the germ layers. This holds not only for the material so far considered—the sea urchin embryo—but also for amphibia and vertebrates in general. I have, however, to refrain from details.

In the progress of differentiation mechanisms arise that stabilize the attained differentiations. These mechanisms may act by repressions to some extent analogous to those found in bacterial systems, cf. [11, 20]. As well known, one has here been able to distinguish two kinds of genes, the structural gene and the regulating gene, the latter operating by production of repressor. Just as "structural" genes underlying for example the animal and vegetal pathways are activated in the course of early embryonic development, regulating genes may also gradually be activated. The repressors produced may act at the cytoplasmic or the nuclear level.* The latter kind of repression may possibly be realized in the work of Briggs and King, cf. [4], dealing with transplantations of nuclei from embryonic nuclei of frog into enucleated egg cells. Such nuclei are able to promote

* This may be the mechanism of "canalization" in the sense of Waddington [29].

development of the egg but in the late gastrula or in the neurula stage restrictions occur that have been demonstrated particularly in endodermic nuclei. With respect to stabilization a variety of different conditions must be expected according to the organism under consideration. In vegetalized sea urchin larvae islets of ectoderm may, under certain conditions, arise within the vegetal endoderm demonstrating a late revival of apparently extinct pathways [22]. Certain forms of regeneration in adult organisms may mean a similar revival.*

No doubt the views established in microbiological research would allow a unitary conception of the early labile embryonic determination and of the later stabilization which still leaves the door open for revival of suppressed pathways. To get the firm basis for generalizations of this kind an intensified interaction between morphogenetic, genetic and biochemical studies will be necessary.

My rapid account on differentiation started from a rather special case. Nevertheless it may have demonstrated some of the principles and problems involved in this function that results in a gradual unfolding of a multitude of structures. The function is based on the structure of the genetic system but without the interactions that arise during the development the normal pattern of differentiation would not be realized.

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The Central Problems of the Biochemistry of Cell Division

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The frame of reference for any consideration of cell division is the whole cell. Entering division, it acquires poles and an equator. When we contemplate the reproduction and distribution of the genetic equipment in the mitotic cycle, we can no longer confine ourselves to questions concerning the molecular character of genetic information, but now must consider how it is packaged into chromosomes. The problems of the chromosome involve us in behaviour and movement, and not merely the control of biosynthesis. The movements are rapid and orderly; the distances travelled are very long by molecular standards. The cell as a whole divides itself in a way that is consistent both in timing and in geometry. At the end of the cycle, we have two full-fledged cells, each with the capacity for living its own private life, where previously we had one. The whole operation of cell division is not only a large-scale operation, being played on a cellular stage in micron dimensions, but is also a purposeful one in an intelligible and unpangllossian sense.

Having to deal with these problems of large-scale structure, of large-scale polarity, with complex but sensibly co-ordinated movements, and with precise timing, the student of cell division does not have to be reminded of the need to correlate Biological Structure and Function, the theme of this Symposium. The correlations are built into his every problem.

I hope that this introduction, which has been intended as a descriptive characterization of the problem of cell division, is not interpreted as an apology for its difficulty or complexity. Of course, cell division is complex in a sense in which a single biosynthetic step, for example, is not, but it is an analyzable complexity. We can analyze it into unit processes which are not quite so formidable in themselves. Such an analysis has recently been discussed by me [1]. The difficulties are real, but we may take the optimistic view that they arise chiefly from our lack of a biochemistry of structure and of multimolecular phenomena. This could hardly have been asked of the infancy or adolescence of biochemical science, but we may now expect it from its maturity.

It is demonstrable, in a sufficiently lengthy treatise on cell division (cf. [2]), that the problems of cell division are linked in some way to almost all of the biochemical problems of the cell, yet certain events may be regarded as being specifically related to division. Some of these have been partly amenable to biochemical attack or speculation, and these will be outlined.

1. DNA synthesis

So far as we know, the life of an individual cell does not depend on continued synthesis of DNA. With some exceptions that should not be ignored (cf. [3]), the doubling of DNA may be regarded as a preparation for division. One of the truly important discoveries of modern cytochemistry has been the demonstration that DNA synthesis—in cells that divide by mitosis, which includes all plant and animal cells—takes place between divisions, in anticipation of division, and not during the mitotic period. In differentiated multicellular organisms, certain categories of cells do not divide, and these generally do not synthesize DNA, but retain the DNA received from the division at which they arose. Since many such cells can be made to synthesize DNA and to divide under carcinogenic influences or merely by removing them from the organism, their failure to synthesize DNA can be viewed as an inhibition imposed by their environment. If DNA synthesis takes place at all, it generally goes all the way to a doubling of the original amount. In a larger view, the “regulation” or “control” in the sense of a modulation of time or rate or ultimate amount synthesized is not a serious problem for the present. The questions of “control” are: (1) How can DNA synthesis be totally suppressed by a great variety of organismal factors; hormones, immunity factors, etc.; and (2) why does it stop when the original dose of DNA has just doubled? The first question, one predicts, will be solved as a straightforward though profound biochemical problem, involving such variables as the induction of the polymerizing enzyme or of enzymes providing the nucleotide precursors or in terms of direct inhibition of the enzymes assuming that they are always present. There are, however, structural factors of major importance. These express themselves in the fact that DNA synthesis, in cells of higher organisms *can* take place only during the phase of the cell cycle from telophase to the next prophase, when the chromosomes are so thoroughly extended or uncoiled that they are not resolvable with the microscope. It cannot take place during the mitotic period when the chromosomes are coiled into the compact packages by which we recognize them. The condensation of the chromosomes for mitosis is intelligible in terms of the requirements for moving them about. The fact that this condition is incompatible with DNA synthesis explains the discontinuity of such synthesis in the life-history of cells of higher organisms. The recent

evidence that DNA synthesis may be continuous during the life cycle of bacteria creates no paradox; it may merely be telling us that the bacterial genetic apparatus is not required to go through a mitotic cycle.

I know of no reasonable speculation to account for the fact that DNA in cells of higher organisms only doubles, after which synthesis ceases until the cell has divided. If such a limitation is not inherent in the enzyme system it may be referable to chromosomal organization, which will be considered next.

2. Reproduction of the chromosomes

Even if one regards the chromosome genetically as a package of DNA, the problems of cell division draw our attention to packing as well as to its contents, and even to the handles by which it is carried. No one doubts that the chromosome is not only large, but also chemically complex. It contains at least as much protein as DNA, and lipids and RNA have also been included in estimates of its composition.

Some fundamental questions concerning the chemical structure of chromosomes have been under study for 20 years or more without being resolved. One such question, rephrased in contemporary form, is whether a single chromosome can be viewed as an enormously extended DNA molecule. Such a state of affairs would greatly simplify the theoretical structure of genetics, for it would abolish an otherwise necessary distinction between coarse and fine-structural genetic phenomena, and would tend to validate the phage or the bacterial chromosome as a general genetic model. Experimentally, the question takes this form: is the chromosome a DNA continuum to which discrete protein units are attached (cf. [4]), a protein continuum to which discrete DNA units are attached (cf. [5, 6]), an assembly of nucleoprotein macromolecules which are linked to each other by bonds weaker than covalent bonds (cf. [7]), or is it composed of alternating segments of DNA and protein? Unfortunately, there is plausible evidence for all of these views. Quite apart from the elegancies of genetic theory, we need a decisive answer to this simple question before we can make a pointed attack on chromosome *behaviour* in cell division.

A second question concerning the complexity of the chromosomes concerns their fundamental multiplicity. To what extent are they composed of bundles of genetically identical units, representing redundancy of genetic information.

Thus far, each method of attack on the question leads to a different answer. The geneticists prefer that each chromosome be a single element, for if it is a bundle of identical elements the interpretation of mutation becomes difficult in a number of ways. The cytologists and the students of chromosome breakage have preferred a two-unit chromosome and can

adduce convincing visual evidence for it (e.g. [8]). The electron microscopists present us with evidence of a still higher level of multiplicity, though not with immense numbers (e.g. [9, 10]).

There is still a third level at which the complexity of the chromosome must be considered; this involves elements which are not "genic" in the usual sense, but may be consistent functional parts of a given chromosome. One is the nucleolus (and nucleolar substance). In many cells, there are compact nucleolar bodies, associated with definite regions of given chromosomes. In addition, it has recently been shown that a nucleolar substance is associated with other regions of the chromosomes [11]. Functionally, the nucleolar equipment may be regarded as that part of the chromosome which operates at the RNA stage of the DNA RNA protein relationship. (A more specific statement would be difficult to make, and need not concern us here.) A second functional component of the chromosome, and the one that is of crucial importance for cell division, is the kinetochore or centromere. This is a distinct body, associated with each chromosome, which is absolutely essential for its movements in cell division. Visually it appears as the point at which the chromosome is engaged by the mitotic apparatus; more crudely put, it is the "motor" of the chromosome. It is not only essential but, if lost, is irreplaceable. In short, it meets one of the fundamental requirements of a reproducing element. We know nothing about its chemistry nor about any of its mechanisms, but its behaviour is as exact and reproducible as that of any element of the cell.

It has been necessary to summarize the evidence for a chemical complexity of the chromosome in order to raise an important biochemical question of cell division; when can we say that the whole chromosome has reproduced and how does it reproduce? We are asking a question with which Molecular Biology is bound to be confronted: how does a complex and—on a molecular scale—"three dimensional" body reproduce?

I have recently suggested [1] that the reproduction of the *whole chromosome* is carried out by a "generative" method. Starting with a complete chromosome, its DNA first reproduces by a genuine replication mechanism. This is the event of *conception* of a new chromosome. We now have a complete chromosome plus an additional allotment of DNA. The "daughter" DNA now serves as the seed or centre for the *development* of a complete daughter chromosome. This takes time, and will not be completed, according to our fragmentary evidence, until the time of the next following division.

This picture of a generative reproduction of the chromosome is more easily understood from a diagram than from a verbal description (Fig. 1). It gives us a simple reason why the chromosome is fundamentally a duplex structure. In any system reproducing by a generative scheme, where a period of development is required between conception and parturition, we

expect to find two generations in existence at the same time. The double chromosome is not always strictly double; during the period of development it contains two sets of DNA but may consist of a complete parent chromosomal unit and an incomplete daughter chromosomal unit.

This generative scheme of chromosome reproduction seems rather complicated, but no more so than the better known case of the reproduction

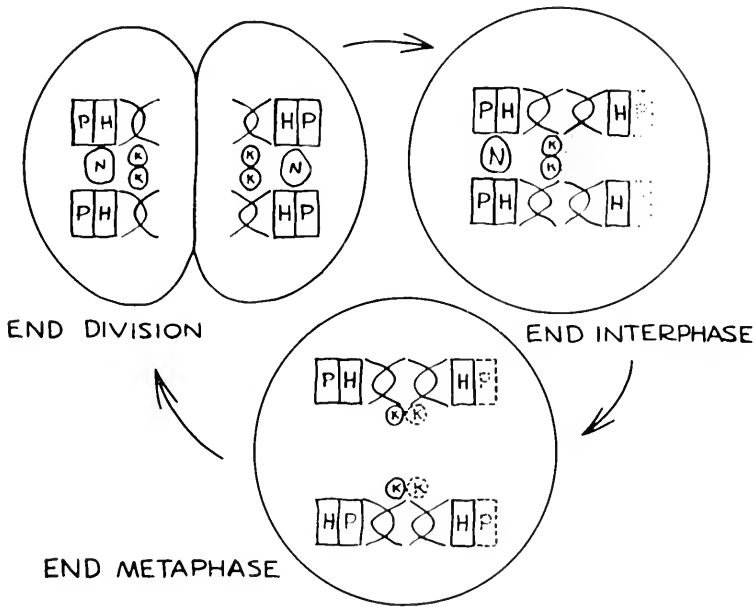


FIG. 1. Diagram of a possible generative plan of reproduction of the whole chromosome. Chromosome is represented as genetically duplex at all times. DNA (symbolized as double helix) replicates during interphase and is simultaneously joined to histone, which may be synthesized at the same time or earlier. Other chromosomal proteins (P) are synthesized or incorporated later. In this version, the complete reproduction of a chromosome strand, including the reproduction and splitting of the kinetochores (K) is not completed until anaphase. If this is so, the division will send one "old" and one "new" strand to each pole. If the reproduction is completed before anaphase, the four units may split at random. The reproduction of the nucleolus takes place during division; the old nucleolus breaks down at prophase and two new nucleoli appear at anaphase.

of bacterial viruses. There, the parent "soma" seems to be rejected entirely, the DNA reproduces to conceive many new units, and the complete units are later developed around the DNA "seeds". To imagine that the whole bacteriophage reproduces in a single step would now seem absurd.

These questions concerning the reproduction of the whole chromosome

may or may not be important for our understanding of its genetic functions, but they may be the heart of many problems of cell division: the way in which the DNA synthesized in one generation is distributed among descendants, the relation between the timing of genetic reproduction and the timing of cell division, the realization of mutational events, and all of the problems of the realization of two fully operational chromosome sets from one. Some of the experimental problems are out of reach at present, but others are simple enough. For example, there is preliminary evidence [12] that some of the chromosomal proteins are in fact made at a different time than the time of DNA synthesis. On the other hand, the histones seem to be incorporated into chromosomes in parallel with DNA synthesis [13].

The reproduction of the nucleolar equipment is rather unusual in that the original material is given up by the chromosome in prophase and two nucleoli, containing at least some new RNA [14], appear at late anaphase. There is no substantial chemical evidence concerning the reproduction of the kinetochores, although there are many interesting cytological inferences.

3. Chromosomes in the mitotic cycle

The structural behaviour of the chromosomes in the mitotic cycle is one of the dramatic events of the life-history of the cell. As everyone knows, the chromosome substance, which is so highly extended and attenuated between divisions that individual chromosomes cannot be discerned, undergoes during prophase a "condensation" which most cytologists attribute to superimposition of several orders of helix formation. The sense of this event is clear enough in terms of the mechanics of mitosis; the genetic material can be transported in compact packages.

A little can be said about the physiological import of this "condensation". As has already been mentioned, the condensed state of the chromosome seems to be incompatible with its ability to synthesize DNA; the evidence for this is quite good. A case can be made for the proposition that the genetic function of the chromosomes, the control of synthesis, is interrupted during the period when they are condensed [1] but we need not discuss this now. Since the prophase coiling of the chromosomes is the most convenient signal that a given cell is committed to division, its initiation and mechanism represent a major problem, and one that has excited considerable and stimulating speculation [15, 16]. Many of the cytological hypotheses postulate various changes in structural composition, such as the packing of the primary genetic threads into a "matrix", but I am afraid that most of what has been said represents ingenious inference. We simply do not possess any solid facts that bear directly on chromosome coiling.

On the other hand, cytochemical research has yielded some solid facts on changes of chromosome composition during the mitotic cycle: (1) There is an "RNA cycle". Chromosomes acquire RNA during prophase, carry it through the period of their mitotic movements, and give it up at the end of the mitotic period [17]. This is not to say that interphase chromosomes do not contain some RNA, but the fact that they acquire more of it and distribute it by the mitotic mechanism raises some interesting speculative possibilities [18]. (2) There is a cycle of changes in staining properties which can be interpreted as the incorporation of phospholipids in chromosomes at prophase and its release from the chromosomes at the end of the mitotic period [19]. (3) The chromosomes lose their nucleolar substance, including an unknown component identified by its reactions with silver, during prophase and reacquire it at telophase [11]. Various inferences can be made about these changes, but none of them is very compelling as yet. All we can say, and it is not trivial, is that the mitotic cycle does involve some important changes in chromosome chemistry.

4. The mitotic apparatus: general

Once the chromosomes have reproduced, the problem of mitosis is to separate the daughters and to collect them into two equivalent nuclei. The astoundingly precise events can be described in a formal way: (1) There are two poles; (2) Each of a pair of sister chromosomes may be engaged by (or "attracted" to) one pole and the two may not be engaged by the same pole. The realities are, fortunately, embodied in a definite structure, which we call a *mitotic apparatus* [20].

The general structural features of the mitotic apparatus are these:

(1) It is a "solid" coherent body, having properties sufficiently different from the rest of the cytoplasm to permit its isolation, which will be described below.

(2) It is loosely describable as a rather unstable gel in which there are oriented regions or structures which are observed as fibres, both at the submicroscopic and microscopic levels [21]. In contrast with the rest of the cytoplasm, it does not contain mitochondria or other larger particles, but it does contain smaller particles similar to those seen elsewhere in the cell [22].

(3) The "fibres", observed either in fixed material or in living material with the aid of polarization optics, appear to include *specific* connections between chromosomes and poles. Visually, it would appear that chromosomes are engaged to the poles by these chromosomal fibres and are "pulled" to the poles by them. At least, the fibres do predict the paths the chromosomes will follow.

(4) In animal cells at least, and conceivably in all cells dividing by mitosis, the poles are not an abstraction, but are represented physically by particles called "centrioles". The polarization of mitosis by these remarkable particles depends on their power of self-reproduction and on the fact that they move apart, following their reproduction, in a definite way that is superficially describable as a "repulsion". The movement is almost certainly not an actual repulsion, and does not follow an inverse-square relationship [14]. While the reproduction and movement of the centrioles is a major problem in the analysis of mitosis, I shall say no more about it here, but will refer to a recent publication of ours [23]. There is a lot to say about centrioles, but not as chemistry.

In the above summary, I have not discussed the progress that has been made in the electron-microscope study of the mitotic apparatus. This has been reassuring (cf. [24]) to the extent that it has confirmed the "existence" of the reasonable structure that had been inferred from accumulated cytological knowledge, but does not necessarily make life simpler for the chemist with his instinctive homogenizer.

5. Isolation of the mitotic apparatus: the stability problem

The mitotic apparatus is a large body, clearly seen in living cells of many kinds. Sometimes it would seem to occupy a very large fraction of the cell's volume. By using eggs of marine animals, which may be obtained in mass quantities and which divide synchronously following fertilization in the laboratory, we may obtain sufficient material for the isolation of the mitotic apparatus for chemical study. The difficulty of achieving such an isolation arises from the fact that the apparatus is so unstable; if we break open the cell in any of the media that are so satisfactory for other sub-cellular structures, the mitotic apparatus simply falls apart.

It now seems to me that the instability of the mitotic apparatus is perhaps the most interesting of all the problems of its structure, and perhaps holds the key to many other problems of cell structure which we have been compelled to ignore. I shall return to this point in a later section. Experience other than attempts to isolate the mitotic apparatus attests to its instability. It seems to disappear from the living dividing cell, sometimes reversibly, under many chemical treatments, under high pressures, at extremes of temperature, etc. It loses its characteristic orientation very easily *in vivo*. Its structure and orientation may well be the expression of an equilibrium between dissociated, and oriented-associated molecules, an equilibrium that is sensitive to many variables. Such an equilibrium has been discussed by Inoue [21]. If this is a proper approach to the stability of the mitotic apparatus—and I now think it is—we could hope to isolate it in one of two ways. The easiest, and the one which first succeeded in the

hands of Dr. Katsuma Dan and myself [20], is to stabilize the structure artificially, risking the distortion of some of its chemical properties but at least obtaining it as a pure isolate for brute analysis. In effect, our earlier methods—and the ones on which a good deal of our present information depends—rested on the stabilization of the mitotic apparatus by immersing dividing cells, usually sea urchin eggs, in 30% ethanol at -10° . Following this stabilization, we could free and clean the mitotic apparatus by dispersing the rest of the cell, which did not appear to be stabilized, with various detergents and other dispersing agents. For the second step, we in our laboratory have most often used digitonin, although ATP and urea (unpublished experiments with Dr. Rollin Hotchkiss) were also effective. When I refer to results obtained by these methods, I shall generally speak of the "alcohol-digitonin method".

A second and more demanding approach to a more natural isolation is to attempt to mimic, in the isolation medium, the conditions in the cell which provide for the stability of the mitotic apparatus. This could well be hopeless according to the above-mentioned hypothesis of a dynamic stability. If the cell must be continuously active in some way to sustain the structure, of the mitotic apparatus, then this activity could be mimicked only if it were expressed in some simple terminal product or condition. The approach to such a method depended on the hypothesis (discussed by Mazia [25]) that the molecular interactions responsible for the structure of the mitotic apparatus involved sulphur bonds and possibly S—S bonds. On the basis of experience that will not be discussed here, it was imagined that an intermolecular (—SH)–(S—S) equilibrium might be "poised" in one direction or another by an appropriate SH or S—S reagent. To poise it in the direction of S—S, we chose dithiodiglycol (OHCH₂CH₂S—SCH₂CH₂OH). Whether or not the reasoning was correct—and one must admit that it was somewhat woolly—this line of attack finally was successful. The mitotic apparatus could be isolated in a medium consisting of isotonic (1 M) dextrose or sucrose, 10^{-3} M versene, and 0.15 M dithiodiglycol at pH 6.0–6.3. An important point is that it is unstable if the dithiodiglycol is omitted and becomes unstable even after isolation if this substance is removed. In our more recent work, the sucrose medium has proved to be preferable to dextrose for the purpose of eliminating other cytoplasmic particles; otherwise, I am not sure that it makes much difference which sugar is used.

Thus this method, which I will refer to as the DTDG method, is comparable to those used for other kinds of particles with the exception of the requirement for dithiodiglycol. It must be said that we have not yet compared dithiodiglycol with related compounds. The essential steps of the isolation are these: (1) Sea urchin eggs are inseminated and immediately transferred to a medium of Ca-free sea water, versene (0.01 M), and

mercaptoethylgluconamide ($0.001-0.01$ M). The purpose of the mercaptoethylgluconamide, to which we were introduced by Dr. David Doherty of Oak Ridge, is to block the hardening of the surface layers and fertilization membrane by disulphide formation. The principle depends on discoveries made at the Wenner-Grens Institute. (2) The fertilization membranes are removed mechanically by passing the eggs through a fine

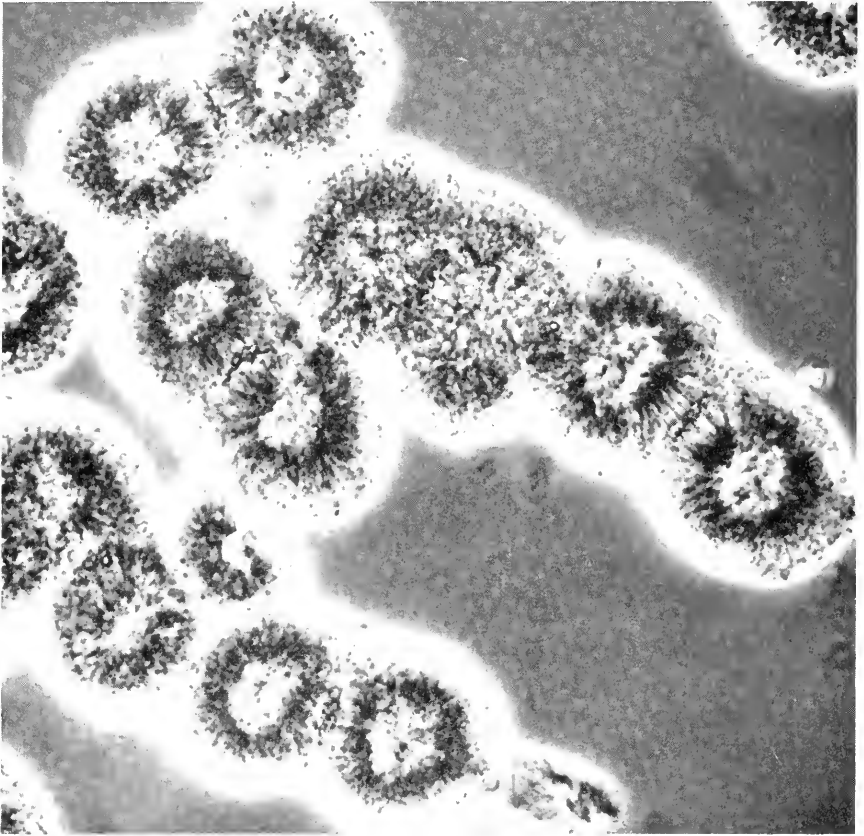


FIG. 2. Mitotic apparatus isolated directly from sea urchin eggs by a new method, described in the text. After isolation, the preparation was exposed to 5×10^{-4} M CaCl_2 , which stabilizes it and sharpens the appearance of the fibres.

silk filter. (3) The eggs are washed in Ca-free sea water. This is very important, as traces of Ca interfere with the isolation. (4) As they approach metaphase, the eggs are washed in a mixture of 9 parts isotonic dextrose to 1 part sea water to lower the ionic strength of the medium. (5) At the desired stage of mitosis they are suspended in the sucrose-versene-D'TDG medium. Gentle shaking by hand suffices to break the eggs and free the

mitotic apparatus, suspended in a smooth homogenate of cytoplasm. (6) The mitotic apparatus may readily be purified and washed by very low speed (200–500 g) centrifugation. Fortunately, most of the smaller particles remain suspended in the dense sucrose medium.

The following properties of the mitotic apparatus as isolated by the DTDG method are pertinent and interesting:

(1) They are extremely sensitive to Ca and Mg, becoming irreversibly stabilized. A Ca^{++} concentration of 5×10^{-4} M suffices to stabilize them. In this form they are beautiful to behold (Fig. 2) because the fibres become highly condensed, but we cannot dissolve them for further chemical work.

(2) They are osmotically sensitive, swelling and shrinking as the sucrose concentration is varied. They disperse at low sucrose concentrations.

(3) They may be dissolved in a number of ways if they have not "seen" Ca^{++} or Mg^{++} . For enzyme studies we dissolve them in isotonic (0.53 M) KCl at pH 8. This has the advantage that the osmotically sensitive yolk particles which are a major contaminant are not lysed and can be separated by centrifugation, along with other particles, probably ribosomes, that are known to be embedded in the structure [22].

It would be brash to suggest that this isolation represents achievement of the goal of obtaining a fully natural mitotic apparatus. A more limited objective was to obtain the mitotic apparatus in a form suitable for studies of enzyme activity, especially of enzymes concerned with ATP. The methods employing alcohol and detergents did not preserve such activity; while the DTDG method does. A second objective, and indeed a long-term ideal of these studies, was to obtain the isolated mitotic apparatus as an effective "model" in the Weber [26] sense of the term. So far, this has failed. We have found no conditions under which the isolated mitotic apparatus will move chromosomes.

6. Survey of the chemistry of the isolated mitotic apparatus

The alcohol-digitonin method provided clean preparations of mitotic apparatus which retained the essential and expected morphological features, and which could be regarded as suitable for the study of some of the major structural macromolecules. Much of the information that has been obtained has already been reviewed, and I shall only list the findings.

1. The mitotic apparatus isolated by the old method consists largely of protein. Conjugation of RNA to the protein has been studied in some detail, but comparable studies have not been made on conjugation of lipid or carbohydrates. There seemed to be little point in analyzing for lipids after isolation with digitonin, and we have tended to formulate the structural problems pretty much in terms of protein chemistry. In view of electron microscopic evidence describing the filaments of the mitotic

apparatus as tubular, and suggesting the presence of structures reminiscent of endoplasmic reticulum in the apparatus in some cases, we are now reconsidering the possible significance of lipoproteins in the mitotic apparatus. The newer method of isolation has made this practicable.

2. The RNA content is relatively high. The last studies [27] give a figure of about 6%. When the major protein components are put into solution, and partly purified, they behave as ribonucleoproteins. At one time, it was thought that adenylic nucleotides predominated [8] but this was erroneous. Better analyses recovered from the mitotic apparatus as RNA having about the same nucleotide composition as the averaged RNA of the whole cell (sea urchin egg) [27]. The present hypothesis is that the structure proteins are in fact ribonucleoproteins. If so, we are confronted with the question of function of the RNA; there is no reason to think that mitotic structure or action involves protein synthesis.

My current speculations about the role of RNA in the structure of the mitotic apparatus take the following form. If RNA carries information regarding amino acid sequences in proteins, perhaps this information may be used for the "recognition" of proteins as well as for their synthesis. Could such a recognition function be involved in the assembly of a structure by association of like molecules? Of course, the RNAs need not recognize proteins; they might recognize each other. If the speculation is unsupported, it does call attention to a question that is easily overlooked: how does genetic information operate in governing the structure of the cell in its larger sense, as well as the structure of its component molecules?

3. By electrophoretic and ultracentrifugal criteria [27] and by immunological criteria [28] the major protein composition of the mitotic apparatus appears to be simple, probably deceptively so. Two or at most three components can be detected, and one of them predominates. It seems to be a protein whose molecular weight is *c.* 315 000 [27].

4. Amino acid analysis [29], data cited by Mazia [30], shows striking similarities between the major protein of the mitotic apparatus and actin from vertebrate muscles. This might be a matter of chance, but might also point to some common properties of structure proteins involved in biological movement.

7. Origin of the mitotic apparatus

It has already been mentioned that the mitotic apparatus occupies a considerable part of the volume of a dividing cell. Analyses of sea urchin eggs and mitotic apparatus isolated from these eggs shows that the mitotic apparatus represents an investment of at least 10% of all the protein in the cell. If the compositional studies cited above are not entirely deceptive, this is mostly protein of one or a few kinds. The mitotic apparatus is not

seen in cells except when they are dividing. The question is whether this amount of structure protein is made as the mitotic apparatus is formed or is made earlier and assembled at the time of division. For the sea urchin egg, the answer seems to be that it is preformed. This was shown by H. A. Went [28] by immunological means. He has demonstrated that the egg before division contains all of the antigens that can be recovered from the isolated mitotic apparatus.

While such a finding, if general, would suggest that the actual formation of the apparatus is a problem of assembly and not of synthesis, it does not follow that the synthesis of structural protein for the mitotic machinery is not one of the important problems of the biochemistry of cell division. The egg is a special case, a cell which is provided with enough proteins, including enzymes, for a long period of development and it undergoes little or no net growth. In a growing population, each cell would have to provide the protein for the mitotic apparatus of the next division, or at least half of it if it "inherited" half from the previous division. But it would be important for our thinking about the control of cell division if the protein of the mitotic machinery had to be synthesized in anticipation of a future division. As Swann [31] has pointed out, the diversion of proteins and protein synthesis into or from the formation of the mitotic apparatus may be an interesting factor in differentiation and the control of division.

8. Thiol chemistry and cell division

The alchemists never succeeded in transmuting sulphur into gold, but the biochemists may yet do so. It is an extraordinary fact that theories in which thiols played a central part have been prominent in discussions of the biochemistry of cell division ever since there were such discussions. One need only cite Louis Rapkine, whose work on a glutathione cycle during mitosis [32] was the stimulus to much contemporary work (discussed by Mazia [25], Stern [33]). Among others who early felt that thiol biochemistry somehow lay at the heart of the cell division problem was Hammett [34], and there were others. In recent years, the study of what we in our laboratory call the "Thiology" of cell division—confessing to an ingredient of faith as well as of good works in this line of study—has developed in a number of ways: in studies on metabolic regulations associated with thiols, in studies of the relation between soluble and protein SH in the dividing cell [30, 35, 36, 37], in studies on the interference with cell division by SH compounds [25, 38], in the demonstration of the participation of interesting sulphur-containing nucleotide polypeptide complexes in cell division in algae [39] and in observations on a specific role of sulphur-containing amino acids in the synchronization of division in algae [40].

(A) BONDING OF THE MITOTIC APPARATUS

So far as the mitotic apparatus is concerned, the present picture is confusing, although not in an unconstructive way. The original design of an isolation method by Dan and myself was based on an hypothesis that S—S bonds were involved in the polymerization of macromolecules into a coherent mitotic apparatus. We proceeded first in a seemingly strange way, artificially stabilizing the mitotic apparatus by deliberately making more S—S bonds by oxidation with peroxide, but it did work. When a method was developed which avoided the use of such an oxidizing agent, the isolated mitotic apparatus seemed to be an S—S bonded structure, soluble only by methods which reduce such bonds [30]. Then this turned out to be a partial oxidation artifact, for it was discovered by Dr. Zimmerman in our laboratory that the freshly isolated apparatus which had been given no chance to oxidize could be dissolved by salyrgan and *p*-chloromercuribenzoate. At the same time, Kawamura and Dan [36] showed by cytochemical means that the mitotic apparatus during the stages at which it was forming was strikingly rich in protein-SH, which became less prominent during the terminal stages of mitosis. It would be satisfactory, and would meet all the facts, to suppose the following: (1) That the apparatus contains SH groups in closely apposed pairs, easily oxidized but not necessarily existing as S—S links in the living condition, and (2) That a linkage of unknown character between these vicinal SH groups holds the molecules together in the structure of the mitotic apparatus. Such a link might be split by agents such as salyrgan and PCMB. There is indirect evidence for the occurrence of such thiol, non-S—S, linkages in other situations (reviewed by Jensen [41]) but the nature of the bond is unknown. I take it that most chemists are not happy with the idea of hydrogen bonding through SH, although it has been defended. In general, the idea that intermolecular hydrogen bonding is prevalent in the mitotic apparatus has been an appealing one (e.g. Gross [42]) and it has seemed to some that an hypothesis of extensive S—S bonding was not consistent with the instability of the mitotic apparatus. Thus, the situation was that the hypothesis of an S—S bonded system led to certain positive results but tests of the hypothesis always favoured the implication of thiol groups in some other way. It was a looser hypothesis concerning sulphur bonding that led to the development of the DTDG method of isolating the mitotic apparatus in a more native condition. The speculations involved will be discussed in a later section.

(B) THE THIOL CYCLE

It should have been stressed earlier that the instability of the mitotic apparatus is not merely an inconvenience for its isolation; it is a funda-

mental property of mitosis in the living cell. The mitotic apparatus cannot be observed as an organized structure in the cell when it is not dividing, though certain parts of it concerned with the centrioles may be present. It can be said to appear when it is "needed" and to disappear when its work is done. One way of saying this is that the intracellular conditions permit its stability during the period of division and no longer do so when division is completed. This loose statement leads to a very specific ques-

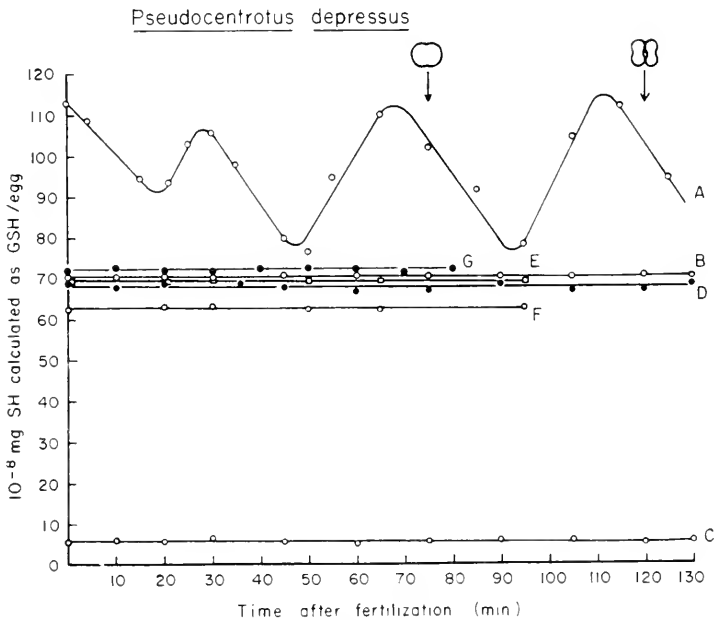


FIG. 3. Fluctuation of a TCA-soluble protein or polypeptide during the division cycle in a sea urchin egg (*Pseudocentrotus depressus*), and non-fluctuation of glutathione. Upper curve; total —SH soluble in 25% trichloroacetic acid. Lines B, D, E, F, G. Soluble —SH after extraction of eggs with saturated ammonium sulphate (B, D), after precipitation of protein from TCA extract (E), and after dialysis of TCA extract. C shows oxidized glutathione (from Sakai and Dan [35]).

tion; is the intracellular environment different during division and between divisions? It was already demonstrated by Rapkine in 1931 [32] that the period of division was characterized by a remarkable fluctuation in the soluble SH content of the cell; the "cycle" involved a striking decrease in soluble SH during the early phases, up to about metaphase, and an increase during the later phases. Rapkine identified the TCA-soluble SH component as glutathione. The situation was confused when attempts to confirm the glutathione cycle as such failed (e.g. [43]), but a brilliant study by Sakai and Dan [35] resolved the problem. The cycle

does exist but it is not a fluctuation of glutathione. Rather, it involves a protein or polypeptide that is soluble in TCA but is precipitable by other protein precipitants (Fig. 3).

Thus it can be confirmed that there is a major fluctuation, during division, of an SH-carrying molecule which may very well be viewed as "environmental" and not part of the structure of the mitotic apparatus. Other and equally interesting fluctuations of the intracellular medium may well be found, but this is the one we now know and can speculate about. One obvious speculation is that the fluctuation is a controlling factor in the mechanochemical operations of the mitotic apparatus, in the same sense that other mechanochemical systems, non-biological systems or "models" of biological derivation, can be driven by appropriate changes in their surroundings.

A second speculation, and one more relevant to the problems we have been discussing, is that the Sakai-Dan cycle, the successor to the glutathione cycle, may account for the assembly and stability of the mitotic apparatus, during the division period, and its instability at the end of the division period. Some years ago I proposed a mechanism of how this could take place by the reduction of intramolecular disulphide, followed by reoxidation to form intermolecular disulphide links [30, 30a]. For reasons given above, I would no longer stress the importance of conventional S—S bonds as such, but the principle may yet hold up in a more refined version involving other intermolecular associations through thiol groups. I will return to this point below.

9. The mitotic apparatus and ATP: the energetics of cell division

Since cell division involves the movement of the chromosomes as well as the formation of a rather elaborate structure, we can certainly assume that it has its price in energy. Attempts to assess this price as an excess oxygen consumption have led to the conclusion that it is probably not very great, but in any case the payment does not seem to be made during the visible phases of division but beforehand. An increased oxygen consumption during division itself is not observed; indeed, Zeuthens' extensive experiments (summarized by Zeuthen [44]) show a slight decline in respiration during the division period. Similarly, inhibitors of respiration, glycolysis, or oxidative phosphorylation do not block division once mitosis has begun, but can prevent it if imposed before a "point of no return" just before the active phases of division. These findings have led to the valuable hypothesis of an "energy reservoir" (Swann [31] and earlier). In some kinds of cells, such an energy reservoir has not yet been detected as a pool of a known high-energy compound. In one case, the

Tetrahymena cell which divides by a mechanism different from ordinary mitosis, Plesner [45] has demonstrated a build-up of nucleoside triphosphates in anticipation of division. As I have pointed out elsewhere [1], the principle of an energy reservoir for division need not necessarily be interpreted in terms of a tangible pool of high-energy compounds, and in fact there are some difficulties with this simple view. Another possibility is that the mitotic apparatus itself is the energy reservoir in the sense that it is assembled in an activated form, and is driven through its manoeuvres by environmental changes such as the SH cycle discussed above.

Another and simple way of attacking the energetics of cell division has sound precedents. This is the examination of its reactions with ATP or other conventional energy sources. No event in the history of the biochemistry of muscle contraction was more portentous than the discovery by Engelhardt and Ljubimova that the proteins involved in contraction included an ATPase activity, even though the outcome was not as simple as might have been hoped. It is natural to ask the same question of the mitotic apparatus, and this became possible when the DTDG method became available. No ATPase activity could be obtained with the mitotic apparatus isolated by the alcohol digitonin method. The studies with the new method were begun by Dr. R. M. Iverson and completed by Dr. R. R. Chaffee. Using straightforward methods of assay analogous to those used for muscle and mitochondrial ATPases, the following information has been obtained, and will be published in full elsewhere. (1) When the mitotic apparatus is isolated and purified by the DTDG method, dissolved in isotonic KCl, the supernatant following high-speed centrifugation shows a substantial ATPase activity. The sediment, representing the particles associated with and embedded in the apparatus, also shows an activity attributable to yolk, etc., but it has different properties with respect to metal activation, etc. It is assumed, for the present, that the activity of the supernatant is that of the "fibrous" component of the apparatus, which is dissolved in the isotonic KCl. (2) The pH optimum is about 8.4. (3) The activity is highly dependent on divalent ions, and Mg^{2+} is three times as effective as Ca^{2+} . Manganese is slightly less effective than magnesium. (4) The enzyme does not split ADP or glycerophosphate. (5) The enzyme is *highly* specific for ATP. It does not split UTP, CTP, or GTP. The splitting of ITP proceeds at a rate half that of ATP or less. I do not know whether there is a precedent for this degree of ATPase specificity.

So far, no other enzyme of the mitotic apparatus has been studied. Whether the discovery of a rather specific ATPase is important for our picture of cell division obviously depends on our point of view. It does seem to link the mitotic apparatus to muscle and certain other motile structures such as flagella, by analogy at least. We are bound to suspect that the energetics of mitosis are conventional enough to involve the

splitting of ATP. On the other side, such a reaction can be only an exiguous part of a complex biochemical picture, and it is not obvious where to turn next.

10. A speculation on the structure of the mitotic apparatus and on cellular structure

The instability of the mitotic apparatus is remarkable, the more so when we consider that its job is to move massive chromosomes over long distances. As further evidence of chemical instability, I may cite the experience of those who have attempted to fix it for electron microscopy with osmium tetroxide or other conventional fixatives. While beautiful results have been obtained in some cases, there are others in which fixation is capricious and still others where it seems to be impossible to preserve fine structure. It is suggested that the mitotic apparatus, especially in larger cells, will not always "stand still" long enough following damage to the cell to be properly fixed before disintegrating.

As we have seen, the idea that the mitotic apparatus was bonded through protein-sulphur has had a certain predictive success, but the theory that it was a simply vulcanized system, bonded through conventional and stable S—S links, has not been substantiated. In the development of the dithiodiglycol procedure to stabilize the apparatus for isolation, we turned to a more dynamic conception of sulphur bonds. This was founded on the growing body of evidence (reviewed by Jensen [41]) that (SH)-(S—S) interchange existed and was perhaps a common phenomenon. The mitotic apparatus was viewed as a massive aggregate in which there were numerous pairs of S atoms located close to each other, and in which S-to-S linkages were opening and closing all the time. As a statistical disulphide structure, its stability would depend on the probability of the existence of a sufficient number of S-to-S linkages at a given time, and it was imagined that this probability could be "poised" at a given level by introducing, in the total system, and S—S compound such as dithiodiglycol. This would influence the level of S-to-S linkage in the protein, acting not quite as a conventional stoichiometric oxidant but as a kind of "buffer" determining the trend of electron flow to and from the protein-SH. Such a view may be outrageously naïve, but it was in fact the basis for the isolation of the mitotic apparatus with dithiodiglycol.

This is not the place to review the body of evidence concerning disulphide interchanges. They can take place in systems containing SH and S—S [46, 41] and can take place between two S—S compounds under the action of ionizing radiation [47]. The point is that a structural system based on S-to-S interactions between proteins can be viewed as a dynamic, sensitive, and unstable one given the right conditions. One imagines that

in these interactions the mitotic apparatus conditions are governed by the fluctuating of thiol-disulphide systems such as the protein of the Sakai-Dan cycle, and that the success of the dithiodiglycol method depends on a mimicking, and no more, of such a system in the living cell.

Perhaps we may speculate a step further. If the mitotic apparatus is such a dynamic thiol-disulphide system, is it not possible that the protein-to-protein interaction includes not only S—S bonds and SH groups in a state of dynamic interchange, but also sites where pairs of SH groups are only half-oxidized? It is likely, from the Michaelis principle of two-step oxidation that such intermediates have at least a transitory existence. Is it conceivable, in a structure composed of so many interacting molecules, that there is an appreciable number of such sites at any given time, and that they are a factor in the stability of the mitotic apparatus *in vivo*. This speculation would view the apparatus as having some of the properties of a gigantic free radical, and this is something that we hope to test. For the time being, one speculates in this way because everyday experience with the apparatus shows: that it is high in protein SH, at least after fixation [36], that it behaves in isolation procedures as a structure that depends on sulphur-to-sulphur links, and yet it certainly does not fit our image of a stable S—S bonded structure. This proposal cannot be defended in any rigorous way, but I wished to mention it here because it is in fact the predictive basis for our current work on the mitotic apparatus.

These problems of stability are not confined to the mitotic apparatus, but to other structures of the cell whose existence is inferred for good reasons but which do not assert themselves either after biochemical isolations or common electron-microscopic fixation. Examples are the gel states of the cytoplasm studied by the late L. V. Heilbrunn and his school and the structure involved in intracellular streaming, discussed in this symposium by Robert Allen. It will not be surprising if the structure of the cell does, after all, include a level of intermolecular organization so dynamic and so sensitive that it has escaped our rather violent direct attacks so far. Such ideas of a "protoplasmic" organization, popular in an earlier era of cell biology but often rejected as being beyond experimental consideration, may yet become accessible to test.

II. Concluding comments

The first step in building a bridge is to span a chasm with a simple cable, and this is what is called for if Molecular Biology is to come to grips with the uncomfortably complex problems of the whole cell such as cell division. An individual experimenter can ignore such problems of the higher levels of cellular organization for the benefits of working with clean

and simple systems, but biology as a whole cannot afford to do so. In this essay, I have tried to point out some links between the formidable problems of cell division and the existing trends of Molecular Biology and Biochemistry.

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Discussion

CHARGAFF: I am wondering about the composition of the mitotic preparations which you have made in the presence of lead thioglycol, in this case without the use of detergents, you presumably do not move very much of the mass. Do you find lipids in the spindle preparations?

MAZIA: I feel certain that we do recover lipoproteins, but chiefly because of our difficulties in sedimenting the proteins of the dissolved mitotic apparatus by procedures that gave good patterns with the product of the older method. Obviously, your question cannot be answered until we have used chemical and ultracentrifugal methods that are appropriate for studies of lipoproteins.

CHARGAFF: Dr. Murray and I studied the effect of colchicine and its reversal by inositol on the metaphase arrest. We got the impression that the mitotic apparatus consisted in part of lipoprotein which after application of detergents you wouldn't expect to find any more. The second question I have concerns the state of the sulphur. Do you have any cysteine determinations on your preparations? What form would the sulphhydryl exist in?

MAZIA: Yes, we have found one cysteine per 18 000 units of molecular weight. This isn't very much, but neither is the mitotic apparatus very stable.

MITCHELL: Could you tell us more about the occurrence of the special proteins of the mitotic apparatus during the resting phases of the cell? I imagine that what you said in the earlier part of your talk—that these proteins are probably always present, or at least are present in the unfertilized egg—means that we do not have to postulate the synthesis of a special supernumerary apparatus for division.

MAZIA: In the immunological studies it was found that the antigens characteristic of the mitotic apparatus were present at all stages of early development

but could not be detected in those adult tissues—gut, lantern muscle, and mature testis—which we were able to study. The optimistic view of these results is that the presence of the proteins of the mitotic apparatus is an anticipation of division, that they will be present in cells that will divide in the future but not in cells that will no longer divide. Thus, they are already present in the ovary, presumably in ripening oocytes, but are no longer present in the mature testis, in which all of the maturation divisions are over for a long time to come.

A problem which has not yet been resolved satisfactorily is whether the proteins of the mitotic apparatus are related to those of cilia and flagella. The common denominator, of course, is the homology of the centrioles around which the mitotic apparatus is organized, and the basal particles of the cilia and flagella.

PETERS: I find this enormously interesting and it does seem that this conception that Dr. Mazia has given us clears up one of the main difficulties in thinking about the cytomosaic (cytoskeleton), but of course we have still in front of us the awful question as how can this become integrated from the cell surface?

MAZIA: I would like to have developed a further speculation concerning the paradox of the existence of so much RNA in the mitotic apparatus. We still have no reason at all to think that this structure is concerned with protein synthesis, and we have reason to think that a substantial amount of RNA is associated directly with the protein (or lipoprotein) making up the "fibrous" structure. If the RNA plays a specific or information-carrying role, and if this is not concerned with protein synthesis, I wonder whether we could visualize it as a "recognition RNA". If molecules are to associate with each other to form an orderly structure, we do have a problem, long recognized, of specific interaction or "recognition". One general view of genetic action is that it dictates specificity at the level of cell structure as well as at the level of the structure of enzymes. Could not the genes dictate structure, communicating information as conjugated RNA, to the molecules of which structures are built?

CHARGAFF: Have you examined the reactivity of the isolated fibrous protein with anti-bodies prepared from proteins of the muscle cells?

MAZIA: We have done the experiments you suggest, comparing the mitotic apparatus of sea urchin eggs with extracts of the lantern muscles of the same species of sea urchin. The results were negative, but could possibly be explained away by problems of diffusion of the muscle proteins in the agar gels used for the precipitin tests. However, Holtzer and colleagues have obtained negative results when they attempted to stain the mitotic apparatus of chick cells in culture with fluorescent antibodies against chick muscle proteins.

Studies on the Cellular Basis of Morphogenesis in the Sea Urchin

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The task of developmental physiology is not only to elucidate how the cells in an embryo become biochemically different from each other, e.g. how some cells get the capability to produce and to accumulate heart myosin, cerebrosides, glucose 6-phosphatase, rhodopsin, etc. We also want to understand how the cells become arranged into various well-organized complicated organ structures such as hearts, brains, kidneys, eyes, etc., which in turn are integrated to form whole organisms.

As a point of departure in our attempt at an analysis we may state that morphogenesis of the organs and the organism as a whole reflects the molecular events going on in its cells. We are permitted to make this statement for many reasons, e.g. treatment of an embryo with agents which interfere with the physical and chemical events in the cells, also brings about characteristic alterations in the anatomical development of the embryo. As an example I may mention that *o*-iodosobenzoic acid, which is capable of oxidizing certain SH groups, suppresses the development of the entomesodermal elements in the sea urchin larva, which therefore only develop into an ectodermal vesicle. A cytosine analogue, 2-thio-5-methyl cytosine, has the same effect. Furthermore, it is generally accepted that genes exert their action by determining the kind of enzymes a cell can form, and we begin to look upon the hereditary morphological deficiencies as the result of disturbances of cellular metabolism.

The problem we are confronted with is to define how the molecular events are translated into organ structures distributed according to a characteristic and reproducible pattern. We may approach this problem from many different directions. One way is to describe the metabolic pattern of cells in different presumptive organ regions in the embryo. But, even a detailed biochemical dissection of an embryo may—I fear—fail to answer our fundamental questions about morphogenesis, e.g. how a certain part of the blastula wall invaginates to form an archenteron and how the archenteron becomes subdivided into coelomic sacs and other derivatives.

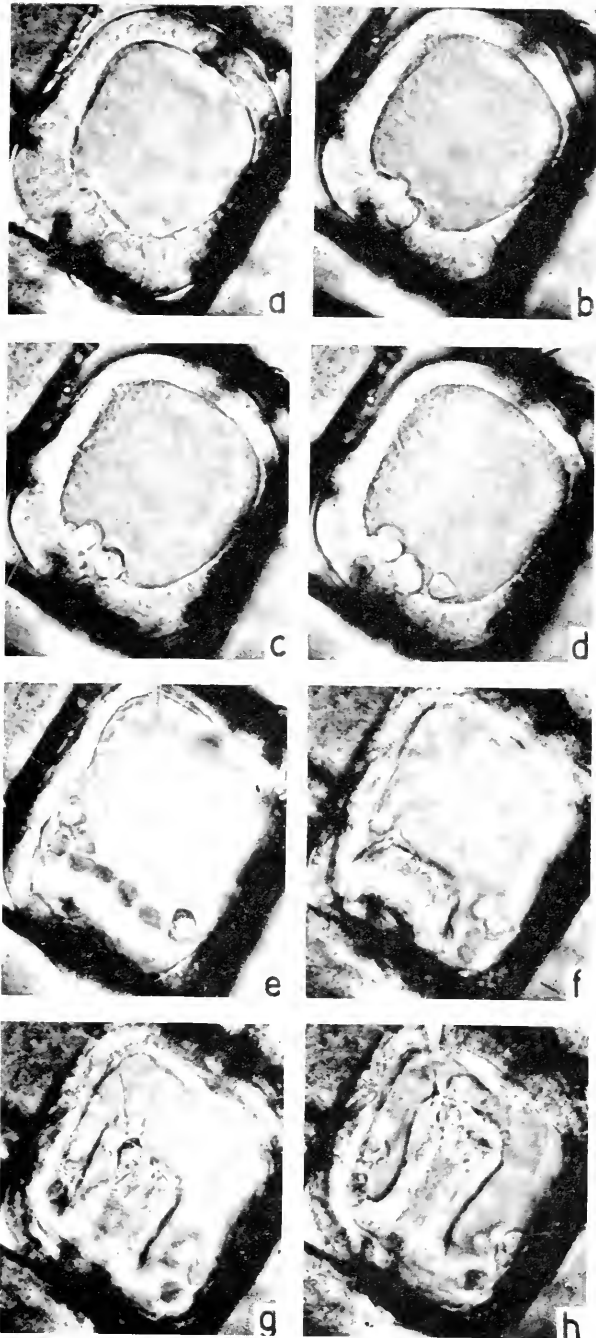
One reason for this apparent difficulty to bridge the gap between the molecular and the organ level is probably differences in language, concepts, and slogans used by the workers in these two fields. Therefore, in order to find a common point where anatomy and molecular biology can meet, it seems logical to try to reduce the complex processes at the organ level into morphological activities of individual cells. Could we, for instance, show that the moulding of a certain organ shape depends upon the formation of pseudopods, upon changes in the adhesive properties of the cells, etc., then we could begin to discuss the molecular background for these cellular phenomena.

The translation of the phenomena at the organ level to a cellular language is greatly facilitated by the use of time-lapse cinematography. The sea-urchin larva is a suitable object for such a study, as it is transparent enough to allow observations of the morphological activities of all its individual cells. Furthermore, its anatomical organization is not too complicated, and finally, much is known about the biochemical differentiation of its cells.

The application of time-lapse cinematography to the developing sea-urchin larva raises some technical problems, as the larvae swim around. We have, however, been able to overcome this difficulty by catching the larvae in the meshes of a nylon net to which crystals of calcium carbonate have been attached. The crystals make small indentations in the larvae and keep them in a constant position without interfering with their normal development [1]. I will try to give some examples of the results we have obtained with this simple technique used in combination with conventional time-lapse filming.*

* The photographs in this paper are all reproduced from our 16-mm. reversal films of developing larvae of *Psammechinus miliaris*.

FIG. 1. The formation of the primary mesenchyme and the archenteron in a larva of the sea urchin *Psammechinus miliaris*. *a-d* show the release of the primary mesenchyme cells which is brought about by a pulsatory activity and the resulting change in shape of the cells in combination with a decrease in their adhesion for each other and for the hyaline membrane; in *d* one of the cells has begun to form a pseudopodium with which it migrates along the blastula wall (to the right). In *e* the primary mesenchyme cells have settled down to form a characteristic ring-like structure. *f* shows the end of the primary invagination of the archenteron, which is brought about by pulsatory activity and the resulting change in shape of the cells at the archenteron tip; it also shows the first sign of the formation of a pseudopodium (protuberance to the left on the archenteron tip). *g* and *h* show different stages in the secondary phase of invagination of the archenteron, which is brought about by the contractile pseudopodia formed from the archenteron tip. The time course of invagination is shown in Fig. 3. Time interval between *a* and *h* 6 hr. 48 min. Picture series from a single 16-mm. time-lapse film. Magnification 430 \times .



It is for practical reasons suitable to denote the lowest part of the blastula (the region at the vegetal pole) as zone 1, the next as zone 2, etc. These regions correspond to the presumptive:

1. primary mesenchyme
2. secondary mesenchyme
3. coelomic sacs
4. oesophagus
5. stomach
6. proctodoeum
7. ectoderm

The films indicate that the morphogenetic activity in these zones can be partly reduced to similar types of cellular activities. One of these is a pulsatory activity of the cell surfaces bordering the blastocoel. The

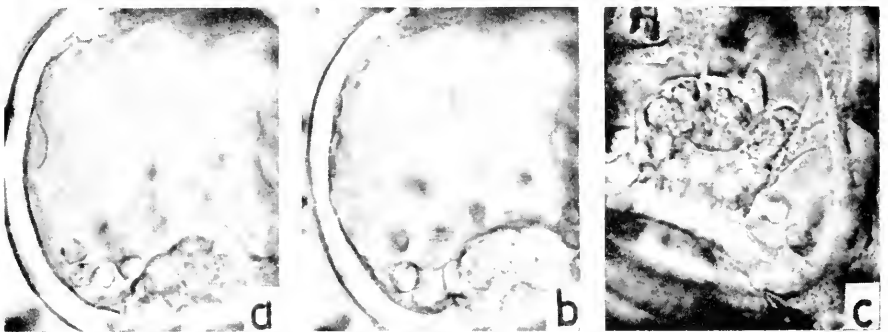


FIG. 2. Further example of the primary invagination (*a* and *b*) and of secondary invagination (*c*).

pulsations involve a centripetal translocation of cytoplasm, which causes the cells to become thicker at their centripetal than at their centrifugal ends. The pulsatory activity is often a forerunner of an emission of thin pseudopods (filopods) from the inner cell surfaces. These pseudopods either collapse or attach to the ectoderm and contract. A third change involves a decrease or increase in adhesion between the cells in the blastula or gastrula wall. These activities may be closely related to each other. It looks, at least, that a vigorous pulsation can be modified into a "shooting" out of pseudopods.

I will try to give some examples how these three changes in cellular activity co-operate in the morphogenesis of the sea-urchin larva:

A. The pulsatory activity of the cells and their resulting change in shape may bring about their release into the blastula cavity. This, however, only occurs if their adhesion to each other and to the hyaline membrane, which covers the blastula surface, is low. The entrance of the

primary mesenchyme into the blastula cavity is an example of this activity, cf. Fig. 1 and [1, 6]. If the cellular adhesiveness remains unchanged, on the other hand, the pulsatory activity and their change in shape bring about an invagination of the body wall. The first phase of invagination of the archenteron and the onset of the evagination of the coelomic sacs from the archenteron tip are examples of such a process, cf. Figs. 1, 2 and [3, 6].

B. The pseudopod activity brings about a strong extension of the invaginated or evaginated regions of the body wall. The second phase of invagination, cf. Figs. 1, 2 and [1, 3], and the extension of the coelomic sac rudiments [3] are brought about by such a mechanism. The bending of

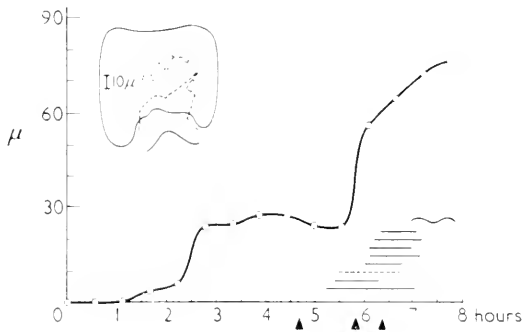


FIG. 3. Diagram of the course of invagination of the archenteron in a larva of *Psammochinus miliaris* (the larva in Fig. 1). Inner height of the archenteron in microns is plotted against relative age in hours, the first stage arbitrarily called 0 hr. The pseudopodal activity is symbolized by the horizontal lines below the curve to the right, each line representing one visible pseudopodium; dashed line: pseudopodium intermittently visible; wavy line indicates direct contact between the secondary mesenchyme and the ectoderm. The schematic drawing to the left gives the appearance of the larva in three different stages corresponding to the marks below the abscissa.

the archenteron tip towards the presumptive stomodaeum region of the ectoderm (Fig. 4 and [2]) is another result of pseudopod contractions. The contractions of the pseudopods will finally pull the pseudopodia-forming cells out from the archenteron tip and a so-called secondary mesenchyme is thus formed. The pseudopod activity finally brings about a rapid migration of the liberated primary as well as secondary mesenchyme cells.

C. The role of a decrease in adhesion between the cells in the wall in the larva has already been exemplified. A strong increase in adhesion between the cells may cause them to increase their contact surfaces so that they become more or less cylindrical. This appears to be the basis for the formation of the ciliary bands and the ciliary plate in the animal pole.

The activities mentioned appear to be released according to a simple

time-space pattern. As an example we may study the release of the pulsatory activity. This activity

starts in zone 1 and brings about a release of the primary mesenchyme cells into the blastocoel;

continues in zone 2 where it brings about the primary invagination of the archenteron rudiment;

continues in zone 3 where it brings about an early evagination of the coelomic sacs;

continues in zone 4 where it may contribute to the morphogenesis (a rounding up) of the oesophagus;

continues (occasionally) in zone 4.

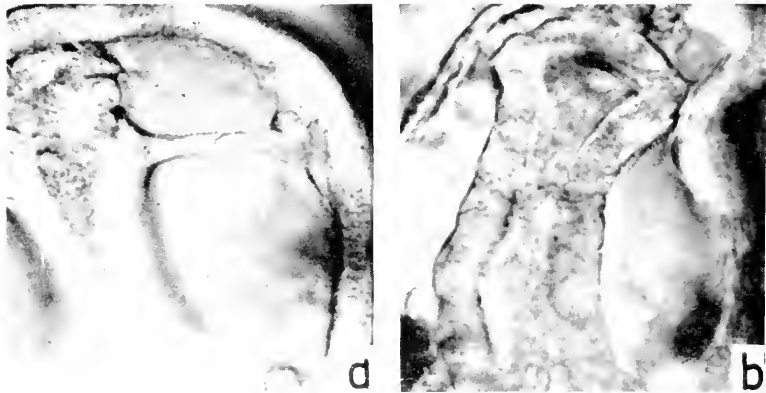


FIG. 4. The bending of the archenteron towards the presumptive stomodaeum. *a* shows how contact has been formed between the archenteron tip and the ventral side by means of pseudopods. The tension in the pseudopods has caused the formation of cones of attachment in the ectoderm and pulled out cells from the archenteron tip, the whole of which will gradually (through the pull of several pseudopods of this kind) be forced to make contact with the ventral side. In *b* the contact has been established. The archenteron tip is sometimes met by the invaginating stomodaeum rudiment, an invagination which recalls the primary invagination of the archenteron.

The pulsatory activity in the other zones generally fails, with some exceptions. It may thus occur (preliminary studies) in the ectoderm where it may contribute to the invagination of the presumptive stomodaeum [2]. It also occurs in the regions between the extending arms and thus contributes to their elongation [4].

The difference in the morphogenetic activity of the consecutive zones is not only a time difference. There also appears to be a decrease in the capability of the cells to pulsate and to emit pseudopods and an increase

in their mutual adhesion and their adhesion to the hyaline membrane. These differences might be summarized as differences in the "strength of their mesenchymal properties" of the cells in the different zones (a provisional and perhaps vague and misleading concept). This intensity

is highest in zone 1: The cells pulsate and lose their adhesion for each other and for the hyaline membrane before the pseudopodal activity starts.

It is weaker in zone 2: The cells pulsate but their adhesive properties only gradually decrease and the cells are only released as a result of a strong pull of their pseudopods.

It is weaker in zone 3: The coelom cells show some pulsatory activity and form pseudopods but they never pull themselves out but remain connected to each other to form cell sheets, the walls of the coelomic sacs.

It is weaker in zone 4: The oesophagus cells pulsate somewhat, but never emit pseudopods. There are, however, contractile elements within the wall of the oesophagus, bringing about its periodic contraction. One can imagine that these elements are, in a sense, equivalent to contractile pseudopods. The main difference may be that they never shoot out from the wall.

It is still weaker in zone 5: The stomach cells may occasionally pulsate but no contractile elements are present and the cells remain connected to a sheet, the wall of the stomach.

This graded change in properties appears to continue within the ectoderm. The cells of this germ layer do not pulsate (with the exceptions mentioned earlier) but the adhesion between the cells is different in different regions. The cells in the thin epithelial sheets can be assumed to have a comparatively low adhesion for each other. In some zones, however, the mutual adhesion increases, and the cells therefore increase their mutual contact surfaces and become cylindrical or more or less hexagonal. This occurs in the most animal region, the animal plate, and in the ciliary band which extends from it. (This rearrangement of the cells is no doubt responsible for the ventral flattening of the gastrula.)

If this interpretation is correct, there is thus a more or less continuous spectrum of morphogenetic properties along the animal-vegetal axis of the larva. The closely packed ciliary cells in the animal plate and the ciliary bands represent one end of this spectrum, the primary mesenchyme cells derived from the zone at the vegetal pole represents another extreme case. It is tempting to relate this spectrum in cellular activities to the animal-vegetal gradients, so familiar to the embryologists, cf. [7].

The film and this review suggest that the ectoderm has a rather

restricted capability for strong deformations. It may change its shape somewhat (e.g. form a ciliary plate and ciliary bands, undergo a dorso-ventral flattening and form arm buds) as a result of a change in adhesion between some of its cells. When it deforms strongly, the forces required are provided by the mesenchymal and mesodermal elements: the extension of the arms, Fig. 5, and the scheidel (the dorsal extension of the ectoderm), Fig. 6, are thus dependent upon the clusters of skeleton forming mesenchyme cells which collect at the tips of the skeleton spicules and push the ectoderm forwards. As a further example, the dilatation of the mouth appears

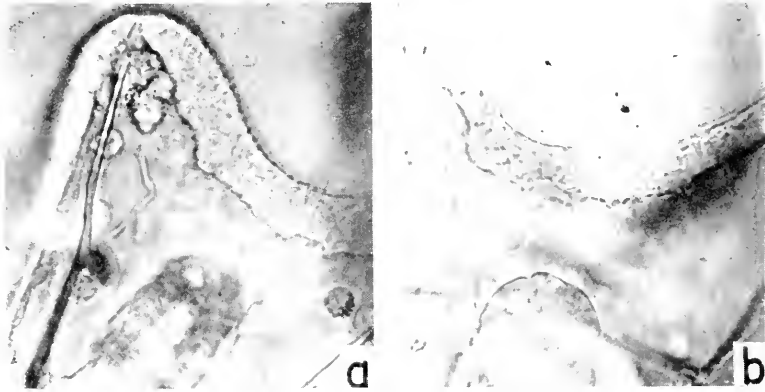


FIG. 5. Anal arms (seen from the anal side) in larvae of *Psammecchinus miliaris* in early pluteus stages. *a*, arm with the typical cluster of mesenchyme attached to and forming the growing skeleton. The extension of the arm rudiment is brought about by the pressure of the mesenchyme cluster. The region between the two arms (right arm outside the picture) shows a pulsatory activity which brings about an invagination. *b*, the pulsatory activity between the anal arms is supplemented by the emission of contractile pseudopodia which, for example, attach to the skeleton and exert a tension which partly causes a release of the pseudopod-forming ectoderm cells.

to depend upon the contractility of the oesophagus. (The expansion of the stomach rudiment into a thin-walled vesicle is also greatly dependent upon a contractility outside the rudiment itself, i.e. is brought about by the hydrostatic pressure generated by the contractions of the oesophagus.) The ectoderm may, however, acquire a pulsatory and pseudopodal activity in certain regions, i.e. in the regions on the ventral side which early have been in close contact with the ventral clusters of primary mesenchyme. I refer to the invaginating regions between the arm rudiments. The invaginations are brought about by pulsatory and pseudopodal activity, Fig. 5, and thus are reminiscent of the invagination of the archenteron rudiment. It may be permitted to suggest that the ectoderm in these

regions has acquired certain mesenchymal properties as a result of its close contact with the primary mesenchyme. One might denote this as a kind of induction.

The ectoderm is, however, not only a toy with which the mesenchyme plays—it is to a great extent the ectoderm which guides the mesenchymal pseudopodia and thus the morphogenesis of the entomesoderm. One may thus say that the ectoderm serves as a kind of *template* for the entomesoderm, but of course not a template in the biochemical sense.

How does the ectoderm guide the mesenchymal pseudopods? The films indicate that the ectoderm in some regions has a high “stickiness” for the pseudopods, i.e. permits the pseudopods to attach strongly. The



FIG. 6. A developing scheitel (dorsal extension of the ectoderm) in a young pluteus stage. The extension of the ectoderm is brought about by the plug of mesenchyme attached to the growing skeleton rod in the same way as in the extending arms, cf. Fig. 4.

stickiness is lower in other regions. There is, in other words, a characteristic pattern of stickiness at the inner surface of the ectoderm. But how do the mesenchymal pseudopods find the areas of high stickiness?

The films show that the pseudopods of the primary mesenchyme are very long and numerous and that they appear to explore the whole inner surface of the ectoderm, cf. Fig. 7. During this apparently random exploration they come in contact with regions of low stickiness as well as regions where the stickiness is high. In the latter case they attach to the ectoderm and contract and thereby carry the cell body in the direction of the sticky region. In the latter case the pseudopods may either collapse—to be succeeded by new pseudopods—or continue their random exploration until they reach a region where the stickiness of the ectoderm is high. The primary mesenchyme as a whole will therefore gradually arrange itself into a pattern which corresponds to the high points of stickiness of the

ectoderm—the “template” [5]. The attachment of the cells to the ectoderm do not appear to be permanent, however. Preliminary observations indicate that the contacts have a rather restricted life time, and the mesenchyme cells may thus change their distribution as a response to a further elaboration of the pattern of stickiness of the ectoderm. I may finally mention that the adhesion between the primary mesenchyme and the ectoderm may be determined by the same factor that determines the adhesion between the cells within the ectoderm: The primary mesenchyme cells thus seem to accumulate in those regions where the ectoderm cells

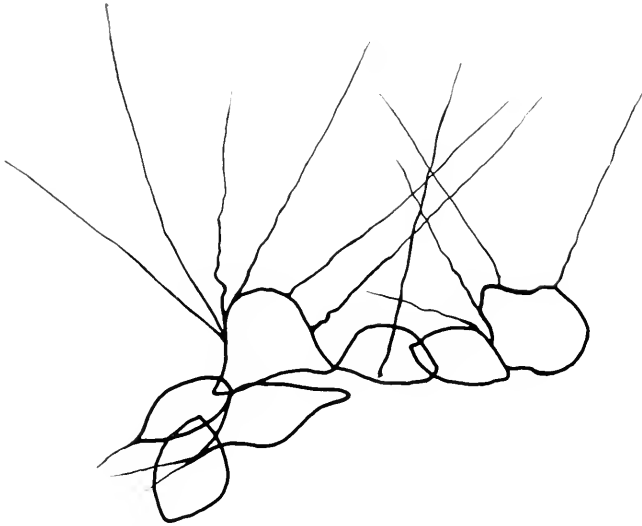


FIG. 7. Primary mesenchyme cells with exploring thin pseudopods (filopods). Drawing from a time-lapse film of a young gastrula. The mesenchyme cells have already arranged themselves into a ring (part of which is seen in the Figure) but still explore the ectoderm in regions far outside the ring level.

will come close together (become more or less cylindrical), e.g. to form ciliated bands. The phenomenon of random exploration is also applicable to the pseudopods formed by the secondary mesenchyme and the coelomic sacs.

As a general conclusion of this brief review we may state that the morphogenesis of the sea urchin larva, in spite of its relative complexity, is not completely obscure but appears to be resolvable into a restricted number of morphological cellular activities, which appear to be released according to a simple time-space pattern, i.e. as a wave proceeding from the lower (vegetal) pole towards the higher (animal) pole. The activities in the different zones bear a certain relationship to each other, i.e. the

difference can partly be reduced to a quantitative change of some basal activities. It may furthermore be permitted to state that pseudopodal elements play an important role in morphogenesis. Such elements are no doubt excellent morphogenetic tools as they not only provide a force which gives rise to translocations and deformations, they also find the suitable direction for the forces—by random exploration—and thereby contribute to a proper integration of the organ rudiments to form an organism fit for survival [2].

It is easy to make a long list of problems for future research. One of the most important problems is how the time sequence for the release of the processes concerned is determined, how the borders between the individual organ rudiments is determined and why they are so sharp, and why the future development of the cells in the different regions diverge. The only point I will make in this connection is that I think that the control of the time-sequence may be a strategic point where the analysis should start. A properly controlled time-sequence may serve as a good basis for a feed-back control of development of less advanced rudiments by older ones.

Finally, may I add a personal comment: I think that the gap between the organ level and the molecular events can be bridged if we try to understand the biochemical basis for pseudopodal formation, pulsatory activity and changes in adhesion between the cells and similar phenomena. Willmer [8] has indicated one way in which such relationships between cellular morphology and the biochemical level can be studied. I refer to his work with the amoeba *Naegleria gruberi* which he is able to transform from an amoeboid cell into a flagellate one, and vice versa, merely by a change in its chemical milieu. And Runnström, cf. [7], has long ago focused our attention on the metabolic gradients in the egg which no doubt appear to be paralleled by gradients in morphological behaviour of the cells. And therefore, as a final personal confession to the participants in this symposium, where much is said about oxidative phosphorylation: the day may come when I, or at least my grandchildren, begin to look upon the discussion between "phosphorylative fans" as something more than a bullfight.

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Discussion

ALLEN: This is a remarkable demonstration of the importance of cell movement in embryonic development. I was particularly interested to observe that there are two kinds of amoeboid movement represented which were ordinarily separated by those of us who are interested in cellular motility. I see formation of both lobopodia and filopodia; the principal difference between these two kinds of pseudopodia is that the pattern of streaming in the lobopodium is that of a fountain, whereas that in a filopodium is two directional streaming. So far we do not know whether these two kinds of movement have similar mechanisms. I wonder if you have looked carefully at the filopodia to find if there is in fact streaming in two directions?

GUSTAFSON: No, I haven't, but during my last sojourn at Kristineberg's Zoological Station we filmed larvae for days at one-second intervals just to investigate the dynamics of the pseudopods.

HOLTER: I was very much interested in your evidence for areas of stickiness that seem to play a determining role in morphogenesis. Isn't there any possibility to determine chemically by means of surface reactions, what would be the reason for this surface stickiness?

GUSTAFSON: I have not tried, but I am very interested to do so.

RUNNSTRÖM: I can tell you that in our experiments very low trypsin concentrations (treatment with 10^{-4} – 10^{-5} % trypsin for 15 min.) induce stickiness of the sea urchin egg. This may indicate a possible role for proteolytic enzymes.

GUSTAFSON: In this connection I may mention that if one treats the eggs with very weak detergent solution one completely changes the pattern of development of the ectoderm: the mesenchyme ring and the main ciliated band form at wrong places and so on. This may give some indication of what lies behind the stickiness in the cells.

PORTER: Your observations suggest that there might be some guiding framework in the blastocoele for the mesenchymal cells; is there fibrous material there?

GUSTAFSON: One can often see a lot of particles in the blastocoele which swim around with great speed. This suggests that there are no rigid structures in the blastocoele at this stage of development. Occasionally, however, one can see particles lined up and vibrating together in a way which suggests the presence of some submicroscopic or at least thin and transparent fibres.

RUNNSTRÖM: As shown by my colleague J. Immers, there are sulphated polysaccharides present in the blastocoele of the sea urchin embryo. In a late blastula stage these polysaccharides become linked to proteins. The migrating cells which Gustafson has studied are in fact surrounded by a coat of a protein-acid polysaccharide complex, a fact that probably is of importance for understanding the behaviour of the migrating cell. If the coat is imperfectly formed the migration of the cells is disturbed or prevented (the latter occurs following pronounced animalization of the larvae).

Cell Differentiation: A Problem in Selective Gene Activation Through Self-Produced Micro-Environmental Differences of Carbon Dioxide Tension

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It is an interesting fact that the subject matter of most of this symposium, i.e. DNA, RNA, ribosomes, mitochondria, etc., concerns the living cell as it was present on this earth a billion years ago, before Darwinian evolution even started. In those dark ages, before there were metazoa of any kind, the primary inventions of protein and nucleic acid

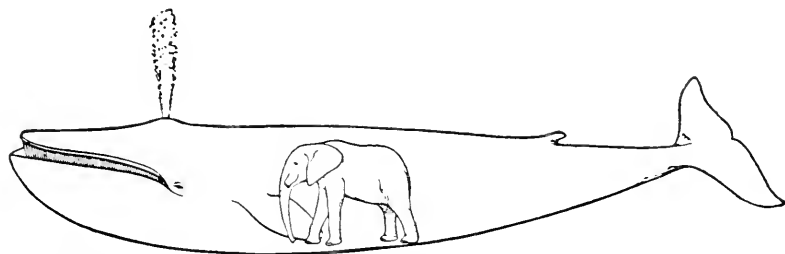


FIG. 1. Relative sizes of a sulphur-bottomed whale—the largest living animal—and the African elephant "Jumbo" (from Lull).

synthesis were combined to effect the miracle of replication. Once this had been achieved, a second series of inventions could begin, inventions by which replication could lead to differentiation and larger and larger multi-cellular organisms arise. *Intercellular chemistry* in other words is needed to explain how thirty quadrillion cells of about a hundred different types co-operate to make a sulphur-bottomed whale (Fig. 1). Embryologically, of course, this vast number of cells is derived by clonal growth from a single fertilized ovum.

Basically, the problem is one of selective gene activation. Since the nucleus of the fertilized egg contains all the genetic information needed to make each of the final differentiated cells present in the adult body, it is clear that only part of this information is used in any one cell. Take, for example, the insulin-secreting cells of the Islets of Langerhans. Sanger

and his colleagues have shown that insulin has the structure given in Fig. 2. A glance at this structure shows that the "one gene, one enzyme" theory must include the incredible fact that "one gene" can contain the 10^{72} or so "bits" of information needed to synthesize such a protein from an amino acid pool. The striking fact is that this insulin-synthesizing gene is present but unused in all the other cells within the body. What is it then that selects which genes are activated where? What are the activating agents? Whence do they come, and how do they reflect the embryo-as-a-whole with all its nearly magical powers of self-regulation?

Even single-celled animals are capable of demonstrating selective gene activation, for Sonneborn [1] has shown that paramecia possess eight different sets of flagellar-protein-synthesizing genes, but the expression of one set automatically inhibits the expression of the remaining seven. It is as if a paramecium were a player piano with eight different tunes stored on rolls within the piano stool. The selection of any one tune for conversion from genotype to phenotype automatically prevents the expression of the other seven rolls.

Most of the gene-activating agents we know today come under the category of maturation hormones, chemicals that activate long-dormant genes during metamorphosis or adolescence. This paper will not consider the various steroid, amino acid and protein hormones that fall into this category, for clearly they are not responsible for the beginnings of development when the complex glands responsible for their manufacture are not yet present. Simple animals such as hydra contain no endocrine glands and indeed no circulatory system, yet they demonstrate cellular differentiation and produce seven different types of adult cells from their original zygote. Clearly there is a chemical progression to development, a series of causes where early effects produce later results almost automatically as envisioned long ago by Aristotle in his famous passage from *De Generatione Animalium*:

"It is possible, then, that A should move B, and B move C: that in fact the case should be the same as with automatic machines shown as curiosities. For the parts of such machines while at rest have a sort of potentiality of motion in them, and when any external force puts the first of them in motion, immediately the next is moved in actuality."

What then is "A" in Aristotle's list, the agent that operates even in the blastula and gastrula? How can the ecto-, endo-, and mesoderm differ so much from each other at such an early date when they were all descended from the zygote just a few cell generations before? My purpose in this lecture is to propose the hypothesis that Aristotle's "A" is in fact carbon dioxide, and that carbon dioxide tension— $p\text{CO}_2$ —is the first self-produced regulator in embryological development.

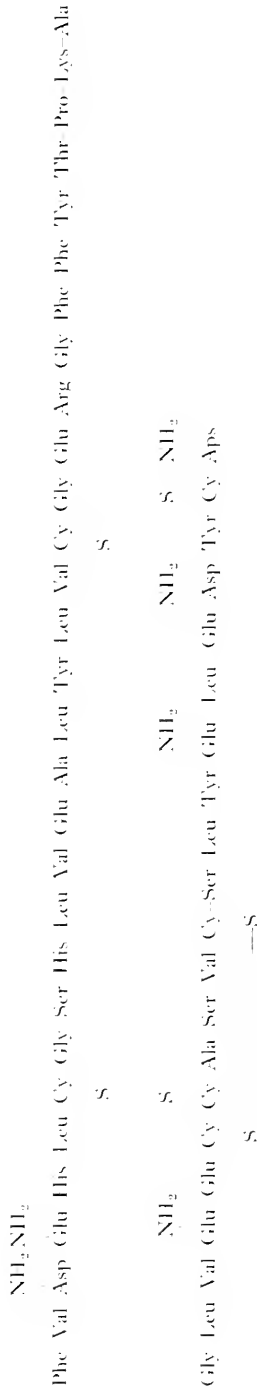


FIG. 2. Structure of insulin.

It is a curious fact that many people react negatively to the mere mention of carbon dioxide tension, saying, "Oh, we know all about CO_2 . There is nothing new in that." Most of them, of course, do not know all about CO_2 , or even about the crucial differences between free and combined CO_2 . What they do remember is the headache they experienced studying this subject in their graduate student days. Such at least was my experience, and it was only when hard experience in the laboratory forced me to the conclusion that $p\text{CO}_2$ was the active variable in my experimental system that I finally sat down and attempted to master the subject both theoretically and experimentally.

This was about 4 years ago. Before that, I had found that hydra differentiated sexually into mature males and females when they were grown in crowded cultures, but did not do so when grown in isolation. Clearly, the question was, "What is in the water of crowded cultures that makes them differentiate along this new pathway, activating these previously dormant genes?" Attempts to take crowded water and use it to turn a single hydra sexual were unsuccessful until it was realized that simple aeration could remove the active ingredient. Here then was a clear-cut system with which to study some of the chemical variables that control cellular differentiation.

Our first finding was that "crowded water" contained less dissolved oxygen than did water in which only single hydra had been grown. This suggested that lowered oxygen tension was the operative variable. Further experiments showed that this was not the case: lowered oxygen tension accompanied sexual differentiation in hydra but did not cause it. Apparently some gas accumulated in the water of crowded cultures that induced hydra to differentiate along the sexual pathway rather than along the asexual. What was this differentiation-controlling gas? Analysis by infrared spectrophotometry, mass spectrography and gas-liquid partition chromatography showed that water from crowded cultures of hydra contained increased amounts of gaseous CO_2 but no detectable amounts of any gases other than those known to be in normal air. Since earlier experiments had shown that no amount of bound CO_2 , such as bicarbonates and carbonates, could induce sexual differentiation in hydra, it seemed necessary to conclude that gaseous CO_2 dissolved in water was the mysterious variable involved. Secondary variables such as ammonia might also be operating in the system, but the ability of free CO_2 to affect cellular differentiation seemed inescapable. This conclusion was strengthened by finding [2] that uncrowded hydra could be turned sexual by growing them in fresh culture water that had been artificially enriched with CO_2 gas (Table I). This experiment has now been repeated in our laboratory seven times and, so far, has always reproduced the published results. I need hardly say that this is a hard rock of fact in a field of variable and con-

TABLE I

CONTROL OF SEXUAL DIFFERENTIATION IN *Hydra* BY $p\text{CO}_2$

Vessel	1	2	3	4	5	6	7	8
Culture water shaken with 100% O ₂ (ml.)	15		14		10		5	
Culture water shaken with 10% CO ₂ and 90% O ₂ (ml.)	0		1		5		10	
Initial $p\text{CO}_2$	0.0%		0.6%		2.8%		5.6%	
Day	Percentage of sexual forms							
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	10	0	0	0
9	0	0	10	30	70	70	70	60
10	0	0	60	50	100	100	100	100
11	0	0	70	60	100	100	100	100
12	0	0	100	60	100	100	100	100
13	0	0	100	70	100	100	100	100

flicting results that is most comforting to an experimenter. Before free CO₂ was recognized, thirteen separate variables had had to be controlled for the 2 or 3 weeks needed for such experiments. Thus, after 4 years of work, I was forced to investigate carbon dioxide tension or $p\text{CO}_2$. How rewarding this has been will appear in the account below, for it not only appears that $p\text{CO}_2$ is the chief variable responsible for sexual differentiation in hydra, but that this factor plays a large and varied role in many other biological phenomena connected with growth and differentiation [3].

Let us start from the beginning. Almost all animal cells respire. Crowding respiring cells together therefore induces partial anaerobiosis in the centre of a cell aggregate that consists of (1) lowered oxygen tension, and (2) increased $p\text{CO}_2$. Two inverse gradients exist therefore in such a cell mass, the $p\text{CO}_2$ gradient resembling the temperature of the sun in that it is highest in the centre and lowest on the outside surface. Both of these gradients may operate at times to create disparity within a previously homogeneous group of cells. Between the two, $p\text{CO}_2$ would seem to be the more likely candidate for Aristotle's "A" because it enters into such a wide variety of cellular reactions as the synthesis of purines and pyrimidines,

the maintenance of oxaloacetate levels as well as playing a vital role in setting the pH of the interior of the cell. In contrast to this high reactivity, molecular oxygen combines almost solely with cytochrome oxidase and that in a manner that is independent of the level of oxygen tension except at the lowest levels [4]. As a possible regulator of cellular differentiation, therefore, CO_2 is a more likely candidate than oxygen; in addition, it has the cybernetic advantage of being actively produced rather than used up with the resulting difference that pCO_2 increases from nearly zero at the surface to its highest point at the centre, while oxygen tension levels do the reverse and hence are far more at the mercy of the over-all environment.

Before continuing, certain facts concerning CO_2 must be reviewed, for they are vital to any understanding of the subject. Thus, it is well known that pH, or concentration of the hydrogen ion, is something quite different from the total amount of acid present in a buffered solution. In the same way, pCO_2 , or the partial pressure of dissolved CO_2 gas, is something quite different from the total amount of CO_2 that may be in a solution in such hydrated forms as bicarbonate and carbonate. Only free gaseous CO_2 is given off by a solution on simple aeration; both free and bound CO_2 are given off following the addition of acid.

Everyone knows the characteristic taste of dissolved free CO_2 gas, for the taste buds of the tongue are uniquely sensitive to this variable as found in beer, champagne and similar carbonated beverages. A vivid demonstration of this fact can be arranged in the laboratory by simply filling a large syringe with gas from a tank of CO_2 and then shaking it with a small amount of water that has subsequently been drawn into the syringe. Since this water sample has been equilibrated with 100% of an atmosphere of CO_2 (760 mm. Hg), it has a pCO_2 of 100% atm. The basic fact is that *pressures* of any dissolved gas equalize whenever gas and water phases are shaken together; if the gas phase has a partial pressure of pCO_2 of 100% atm., then the water phase within the syringe has an equal pCO_2 . It should be noted that syringes are extremely useful because they provide the operator with an adjustable volume that is always automatically maintained at a pressure of 1 atm. In contrast to this simplicity, the *concentration* of free CO_2 dissolved within the water phase changes with both the temperature and the ionic strength of the solution, for Henry's Law states that $[\text{CO}_2] = \alpha \text{pCO}_2$ and both temperature and ionic strength affect the solubility coefficient α .

If the water in such a syringe is now expressed into a small beaker and placed beside a similar beaker of plain water, the demonstrator may remark, "Oh, I have forgotten which is which. I wonder which has the pCO_2 of 100% atm. and which has a pCO_2 equal to that of air?" (0.03% atm.). At this point the onlooker may be challenged to figure out some

way of finding out which beaker is which, using only his five unaided senses. Since no bubbles of any kind are visible in either vessel, no visual difference may be detected. Eventually the onlooker hesitatingly takes a sip from each of the beakers, whereupon a look of certainty crosses his face as he remarks, "This is the beaker that has the high $p\text{CO}_2$. It is unmistakable."

Further experiments can be conducted. For example, does water made equally acid ($\text{pH } 3.7$) with HCl taste the same? (No.) If a $p\text{CO}_2$ of 100% , atm. is unmistakable, can the human tongue detect a $p\text{CO}_2$ of 50% , atm.,



FIG. 3. General view of pilot-plant for *Chlorella* production designed and built by Arthur D. Little, Inc., Cambridge, Mass. Note plastic cover to photosynthesizing trough and large vertical tower for treating culture medium with CO_2 gas.

or of 25% , atm.? Does bicarbonate with an equal total concentration of dissolved CO_2 taste the same? The answers are quickly and vividly obtained, for only gaseous CO_2 dissolved in water affects the taste buds of the tongue in the manner that is specific to beer and other carbonated waters.

The respiratory centre of the brain also contains cells that are sensitive to $p\text{CO}_2$ as a variable different from pH . It is these cells that regulate our breathing in such a way that the percentage of CO_2 in the base of our lungs is held at 5.3% , CO_2 , a physiological mechanism that guarantees that our

arterial blood shall have a $p\text{CO}_2$ of $5 \cdot 3^{10}$ atm. at all times. Many students believe that breathing is regulated by CO_2 as an indirect means of controlling the oxygen in the blood. In fact, the body is immune to changes in oxygen tension within wide limits as may be demonstrated by the lack of any bodily reaction to breathing pure oxygen when at sea-level.

Living cells then react to free or gaseous CO_2 in a manner that is different from their reaction to the bicarbonate ion. This difference between $p\text{CO}_2$ and total CO_2 is fundamental to any understanding of biological crowding effects and stems from the little-known fact that CO_2

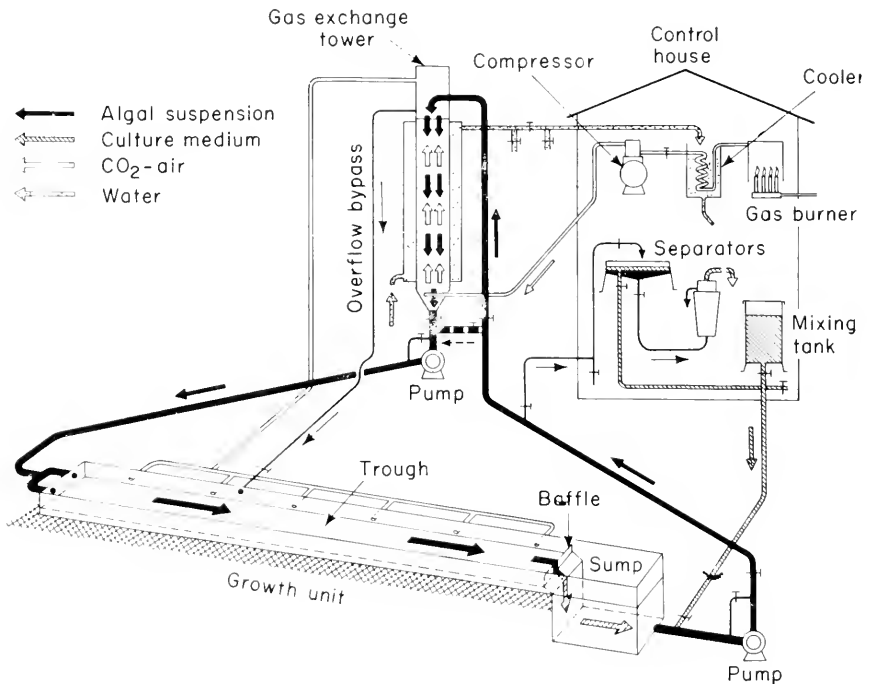


FIG. 4. Diagram of algae plant.

is a fat-soluble gas that is 1.67 times more soluble in lipid solutions such as olive oil than it is in water (σ^0). This lipo-solubility enables the free CO_2 molecule to pass through a fatty cell wall when the bicarbonate ion can not, the cell membrane therefore acting as a semi-permeable membrane that distinguishes between free and bound CO_2 molecules. These facts are well known to plant physiologists, for they have found that photosynthesizing algae can only obtain the large amounts of CO_2 that they require from gaseous CO_2 which can penetrate their cell walls when the bicarbonate can not. Any algae farm therefore has two characteristic features: (Fig. 3)

the large flat culture beds that are exposed to sunlight and the tall towers through which ascending CO_2 gas is bubbled through the descending culture medium that is circulated through the culture beds (Fig. 4). If the bicarbonate ion were equivalent to free CO_2 , then the photosynthesizing troughs would not have to be covered with a transparent plastic cover that reduces the available light but keeps in the vital CO_2 gas. All that would be necessary would be to add bicarbonate to the culture media and grow the algae in direct sunlight.

Jacobs [5] has described an elegant experiment to show that free CO_2 can selectively penetrate cell walls and produce intracellular acidity even in alkaline solutions. He found a flower whose colour changed reversibly from blue to red when it was dipped in alkaline or acid solutions, an internal type of litmus reaction that could be used to determine intracellular pH. When this flower was placed in an alkaline solution that had a pCO_2 of 5%, atm., the colour of the flower changed from blue to red, indicating that the free gaseous CO_2 in solution had penetrated the fatty cell membranes and had ionized inside these cells to form carbonic acid.

Accepting pCO_2 therefore, as an important but unfamiliar biological variable, what are the units used to measure it? The easiest unit I believe is as a percentage of an atmosphere, for almost all biological reactions are carried out near sea-level and hence simple percentages may be used rather than the more confusing pressure units such as millimetres of mercury. Take for example the syringe described above in which water and pure carbon dioxide gas had been shaken together to equilibrium. The water in this syringe has a pCO_2 of 760 mm Hg. but it is just as true, and more vivid operationally, to say that it has a pCO_2 of 100% of an atmosphere. This latter terminology brings to mind the method of making such solutions, for water shaken with 50% CO_2 gas mixtures has a pCO_2 of 50% of an atmosphere; that shaken with 10% CO_2 has a pCO_2 of 10% atm.; and that shaken with 1% CO_2 has a pCO_2 of 1% atm.

This, then, is the simplifying principle behind our nomenclature that allows one to handle concentrations of free CO_2 with the same ease as one handles calcium ion concentration.

We have recently devised a rapid means of measuring pCO_2 that does not involve pH and so avoids many of the complications that have plagued this field [6]. The essence of the measurement is to shake 10 ml. of the sample of water to be tested with an equal volume of air and then to determine how much CO_2 enters the gas phase from the solution. The results of this measurement are simply multiplied by a factor to obtain the pCO_2 of the original solution. In our laboratory this summer, my son and I had side-by-side arrangements for measuring pH and pCO_2 . This enabled us to examine all cultures from both these angles, as well as to vary pCO_2 , pH and bicarbonate concentration relative to one another. Since the

well-known Henderson-Hasselbalch equation states that the concentration of the H^+ ion varies both with the pCO_2 and the bicarbonate present:

$$\text{Concentration of } H^+ \approx \frac{pCO_2}{HCO_3^-}$$

it is clear that any change in one of these three variables will affect at least one of the other two. Picture a metal triangle supported at its centre, on a table, its three corners representing pH, pCO_2 and bicarbonate respectively. If now one of the three corners of the triangle is held firmly against the table, it will remain constant while the other two vary reciprocally like a see-saw. This is the principle of a triad of experiments that we have used to determine which of these three variables is the biologically active one in any given situation. Experimental control of pCO_2 may be effected by (1) exposing shallow Petri dish cultures to known concentrations of CO_2 within a desiccator; (2) injecting closed containers with varying volumes of a culture solution high in pCO_2 that has been previously prepared by shaking it in a syringe with air containing the desired amount of CO_2 gas; and (3) bubbling the experimental culture continuously with air from a tank containing the desired concentration of CO_2 . In my laboratory in Greenwich I have a series of gas tanks that vary from 0.1% to 100% CO_2 . With their aid, almost any desired pCO_2 can be easily and rapidly obtained.

Today, then, tissue pCO_2 represents an old-but-new variable of some complexity. Small wonder that most modern textbooks either ignore the subject or else dangerously oversimplify it. Many people, for example, have been taught that carbonic acid is a weak acid when in fact it is as strong an acid as citric, formic or nitrous acid! [7]. The reason for this widespread misconception is the little appreciated fact that 99.9% of the CO_2 dissolved in water does not hydrate to H_2CO_3 but remains as free gaseous CO_2 [8]. The 0.1% of carbonic acid that does form is a surprisingly strong acid. Taken together, these two facts combine to make gaseous CO_2 equivalent to a weak acid, a simplification that may be legitimate in certain situations but not in others.

Summarizing the physical-chemical facts then, we can say that pCO_2 is a universal biological variable generated by all respiring cells. Being fat-soluble, CO_2 can easily pass through fatty cell membranes and hence unify a cellular aggregate into one overall "field of force". Highly reactive chemically, it enters into many cellular reactions as a direct participant as well as specifically affecting intracellular pH. Present as a gas within the alveoli of the lung, as a dissolved gas within the tissues and finally as a solid within the matrix of a bone or shell, five separate steps are needed to connect all forms of this one metabolite, the final complexity arising when part of this inorganic chain is catalyzed by the zinc-containing enzyme carbonic anhydrase and so is subject to all the variables that affect the

activity of enzymes. Extremely difficult to measure until recently, it is small wonder that most biologists and biochemists have avoided the subject as much as possible and even dismissed it from existence with remarks like, "Oh, we use CO_2 to set the pH, that's all." If this were "all" in fact, then the same pH could far more easily be obtained with one of the many excellent buffers that are not volatile. Yet "setting the pH" with gaseous CO_2 is compulsory in tissue culture (J. H. Hanks [9]):

"The problem of pH control often appears baffling when cell culture work is first undertaken. In view of the simplicity of many buffer systems it seems almost a crime that a gas such as CO_2 in equilibrium with H_2CO_3 and NaHCO_3 should be a major physiological mechanism of pH control, that this system should be essential for respiration and growth, and that it should, at the same time, afford such inefficient buffering action in the working range of pH 7-8. Until such time as man or Maker may provide a substitute, one must be prepared to fight the battle of CO_2 ."

Returning now to cellular differentiation, let us picture nature trying endless experiments at the protozoan level in an attempt to obtain more than one kind of cell with which to build a metazoan animal. Stacking identical protozoa together into larger and larger clusters would automatically expose the central cells to higher and higher levels of pCO_2 . Since fatty cell membranes form no appreciable barrier to free CO_2 molecules, the whole mass of respiring cells would form one large "field of force" whose medullary pCO_2 would be far higher than the peripheral cortex. Such a unifying "field of force" would represent a function of the whole, for if it were cut in two, new gradients of pCO_2 would form within each half just as they do when a pile of glowing coals is divided in two: the centre of each aggregate soon is hotter than the newly exposed periphery.

Suppose a mutant should now arise among these protozoa, a mutant whose DNA behaved differently under high and low levels of pCO_2 : i.e. a protozoan with genetic material that could not be expressed phenotypically except when grown under conditions of high pCO_2 . Clearly such a protistan would replicate until a critical mass was formed in whose centre the pCO_2 reached the postulated threshold for the activation of this additional set of mutant genes. Here then would be a mechanism by which replication could lead to differentiation, and, with further elaboration as during gastrulation, Aristotle's A could lead to B and C.

Thus, it is known that slime-mould amoebae lay down walls of cellulose only when they are buried deep inside the multicellular pseudoplasmodial aggregate. Would it not be a great step forward if it were found that single slime-mould amoebae form walls of cellulose when grown in isolation if they are exposed to increased levels of pCO_2 ?

Many years ago Rachevsky examined the mathematical relations that

would exist around a spherical cell that produced an diffusible metabolite [10]. Figure 5 presents his results as applied to $p\text{CO}_2$. Clearly the curve of $p\text{CO}_2$ is highest in the centre of the postulated cell and drops in hyperbolic fashion towards the periphery. A further drop at the cell membrane then occurs that is dependent on the permeability of the metabolite in question. Since we know that fatty cell membranes are more permeable to CO_2 than to oxygen or even water, this second part of the curve may be essentially eliminated from consideration in the case of CO_2 .

Outside the wall appears a third gradient that I refer to as the "blue

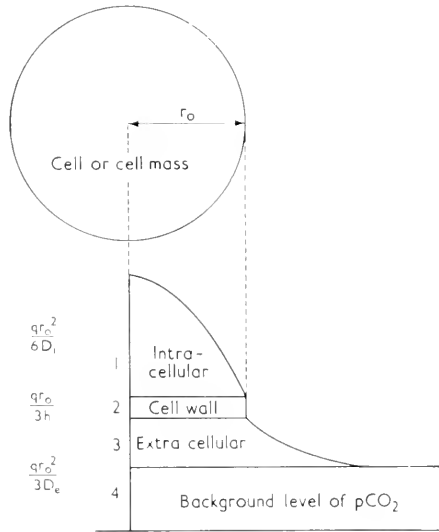


FIG. 5. Gradient of $p\text{CO}_2$ in a spherical cell or cell aggregate: q = respiratory rate; D_i = rate on internal diffusion (or cell streaming); D_e rate of external diffusion (or convectional streaming); h = permeability of the membrane and r_0 = radius of cell or cell aggregate. Modified from Rachevsky [10].

haze" effect, for it makes me think of the quiet lounge of some London club where three older members are reading their newspapers, each member surrounded by a blue haze of pipe smoke that he has produced himself. Clearly any analysis of the smoke within the room that first allowed it to become mixed would not give a correct idea of the smoke concentration to which each club member had been exposed all afternoon. Rachevsky's third or blue-haze gradient therefore reflects the degree of stagnation within the system. Whenever extracellular currents exist, no external gradients can form, while simple stagnation reacts upon a system so as to increase the final level of $p\text{CO}_2$ existing at the centre of the respiring mass.

Rachevsky's fourth factor is the level of $p\text{CO}_2$ in the general background. Human tissues, for example, have a background of 5.3% atm. for this exact level of $p\text{CO}_2$ is carefully maintained in the arterial blood stream by the medullary centre of the brain. Most fresh-water and marine animals in contrast are exposed to a background of about 0.03% atm. [11], for this is the level of $p\text{CO}_2$ that exists in water that has been equilibrated with normal air.

Four separate factors then contribute to the $p\text{CO}_2$ existing at the centre of a spherical cell where the chromosomes usually are found. Of these, the single most important is probably the size of the cell or cell aggregate itself, for here the parabolic curve rises as the square of the radius. Is this the reason that a large cell like an amoeba liquefies its central protoplasm? Certainly no amoeba can build a steep Rachevsky gradient on a permanent basis, for its protoplasm is continually rolling over and thus destroying the geometrical relations that caused it to arise in the first place.

Experimental study of amoebae in our laboratory suggests that their reversible solation-gelation is $p\text{CO}_2$ dependent. Pantin, for example, has used pH-dependent vital dyes to show that the cytoplasm of newly forming pseudopods is decidedly more acid than the older gelled material [12]. We have taken actively migrating amoebae that are extended in thin strands like the horns of a deer and exposed them to 20% CO_2 . Almost immediately, the ends of such staghorns begin to soften and "melt" back into the body of the amoeba so that it soon assumes a uniformly round and spherical appearance. Clearly the normal inside-out gradient of $p\text{CO}_2$ has been abolished by the artificial application of 20% CO_2 from the outside-in. If now this same animal is examined 20 min. later, it will be found to be moving around in normal staghorn fashion just as if it were not still under 20% CO_2 . What has happened in these 20 min.? Clearly the answer is that continued respiration on the part of the amoeba's large mass of cytoplasm has allowed a new inside-out gradient to form even against an outside background of 20% CO_2 ; the inside of such an adjusted amoeba therefore is again softer than the outside and once again it can begin to flow out from a central liquid pool in successive "larva flows" that cool and solidify as they lose their excess CO_2 . Experimental evidence for this view is provided by the fact that this adjustment to 20% CO_2 does not occur under anaerobic conditions. Further evidence is provided by the fact that dinitrophenol does not inhibit normal amoeboid movement even though it is known to uncouple specifically oxidation from phosphorylation [13], and hence inhibits ATP production while allowing CO_2 production to continue. Finally, it should be mentioned that a $p\text{CO}_2$ of 50% atm. permanently liquefies such an amoeba and reduces it to a round sphere that is unable to form pseudopodia. By this view, amoeboid

motion results secondarily from the protoplasmic streaming that was originally designed to aerate even the innermost cytoplasm of a cell. With a cell as enormous as an amoeba, this is, of course, more necessary than usual.

Gradients of $p\text{CO}_2$ are highly dependent on simple geometrical forces such as those of total mass, flattened versus spherical shape and similar changes in the surface/volume ratio of an aggregate of cells. A delightful description of the fundamental character of such geometrical forces is presented in John Bonner's *Morphogenesis* [14]. His conclusion is inescapable: i.e. nature uses these simplest of all considerations to build up progressive complexities during the development of an embryo. The enormous difference between the fate of a blastomere that is separated from other cells and one that is left attached to another blastomere, be it alive or dead, is a case in point. Even a dead blastomere somehow affects its living twin by its mere physical presence, an effect that can at least speculatively be assigned to a distortion of the gradient fields of $p\text{CO}_2$ produced in the living half.

If embryonic differentiation is to proceed along a $p\text{CO}_2$ gradient of the type Rachevsky pictured, it must be permanent and not upset by cytoplasmic streaming as in the body of an amoeba. One means of stabilizing such a gradient is to have the egg cleave progressively into smaller and smaller cells so that the protoplasm at the centre of the mass is locked in place along the over-all gradient that extends throughout the entire mass of respiring cells (Fig. 5). Looked at from this angle, it is not surprising that cell cleavage is the first order of business in the developing embryo, for it prevents cytoplasmic streaming by the erection of cell membranes through which CO_2 molecules may travel but behind which the protoplasmic contents of each cell is locked in place. According to this view, a physico-chemical gradient of $p\text{CO}_2$ would first form as a result of the respiration of the cells themselves. Only gradually would this chemical gradient be transformed into structurally different cells that differentiated in each location according to the micro-environmental level of $p\text{CO}_2$ that existed at that particular site.

John Bonner has emphasized that the embryo uses cell movement as well as cell growth and differentiation to achieve its ends (Fig. 6). Here we encounter such problems as (1) the acrasin phenomenon in which slime-mould amoebae become mutually attractive to each other; (2) why the dorsal lip of the blastopore grows downward and into the hollow sphere of the blastula when it might well grow outward into new space as during budding; (3) why certain epithelial cells sink down below the surface as during the formation of the neural groove.

If cell migration is vital to embryogenesis, perhaps it is because it takes cells that have been programmed one way and then exposes them to another

micro-environment such that "outside" cells now find themselves "inside" as in the case with buried neural tissue that once was external epithelium. The three germ layers of the embryo, in fact, represent the outside and inside of a hollow sphere respectively, together with those most anaerobically placed, the mesoderm that is literally sandwiched between its epidermal and endodermal neighbours. A picture of sequential programming thus arises, the order of the programming being vital in that exposure to high-then-low levels of micro-environmental $p\text{CO}_2$ should

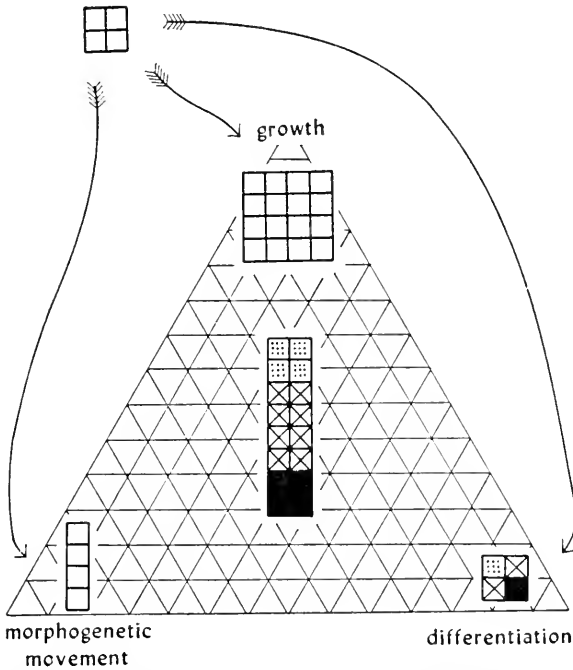


FIG. 6. Diagram to illustrate the interrelations between growth, morphogenetic movement, and cellular differentiation (from Bonner [14]).

cause a different type of differentiated cell from one that has been exposed to low-then-high. As in the education of a young man, exposure to different environments can produce different final results.

Why do cells move from one location to another within the developing embryo? One answer may be that they become positively or negatively chemotactic to $p\text{CO}_2$. Thus, *Chlamydomonas* cells are known to be chemotactic to $p\text{CO}_2$ [15] and even the acrasin phenomenon in slime moulds may be due to the mutual attraction of the washed amoebae owing to their positive chemotaxis towards a high $p\text{CO}_2$ such as that generated by their neighbours. Preliminary experiments in our laboratory have shown that

as little as 5% CO₂ reversibly inhibits aggregation, an inhibition that may be due to the failure of one amoeba to find the CO₂ generated by its neighbour when a high percentage of CO₂ is present everywhere and in no relation to the location of other amoebae. This possibility is further supported by the fact that slime mould amoebae will not aggregate in the presence of bacteria—possibly because these bacteria also generate CO₂ and so drown out the amoeba-to-amoeba message. Like fireflies in the daylight, the background is too high for the “message” to get through.

In summary, embryology seems surprisingly hospitable to various applications of the pCO₂ theory. Not only does it shed light, and suggest experiments, on the meaning of egg cleavage, but it continues to do so during gastrulation and the formation of the three germ layers. Formative cell movements connected with the formation of the heart have been shown by Ebert [16] to begin only after a critical cell mass has been achieved. Looked at from the pCO₂ point of view, such facts become understandable, for the embryo seems to contain its future structure within itself much as a fireworks rocket exploding in the night sky first releases a burst of blue, then red, followed in turn by yellow and green, the fuse of each setting off each subsequent explosion. Rather than looking for some structure-giving external organizer such as was pictured in Spemann's day, we can visualize a sequence of micro-environments self-created by the developing egg as it cleaves into a thousand cells and then invaginates to form the three-layered structure of the gastrula. Spemann's induction of a second embryo is not so miraculous as it first appears, for once started, an inevitable chain of events would proceed as they do when activated by the entrance of a sperm. Indeed the facts of parthenogenesis suggest that the originating stimulus is of secondary importance, for many causes may set off the chain reaction that is embryogenesis. The real miracle is that unwanted embryos do not start more often; perhaps they do as teratomas, those monstrous tumours composed of disorganized bits of hair and cartilage, bone and epithelium.

Space does not permit consideration of the many possible biological roles of tissue pCO₂ in tissue culture, cytostasis, neoplastic growth, sexual maturation in both plants and animal tissues, limnology, etc. Some of these aspects were discussed at the 17th Symposium of the Society for the Study of Development and Growth [3]. Suffice it to say that work for many hands exists within this difficult but rewarding area, work that can aim at reproducing on the level of an isolated cell all the conditions found within a developing tissue, so that such an isolated cell differentiates morphologically just as if it were still surrounded by its normal cell neighbours.

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RNA Synthesis in the Nucleus and RNA Transfer to the Cytoplasm in *Tetrahymena pyriformis*

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Studies of [^3H]-cytidine labelling in *Tetrahymena* have been made in regard to two problems, (1) the transfer of RNA between nucleus and cytoplasm, and (2) the nucleus as the major or exclusive site of RNA synthesis.

In earlier experiments, diverse types of cells have been incubated with [^3H]-cytidine or other labelled precursors of RNA. The first radioactivity incorporated into RNA has consistently been localized in the nucleus [1-7]. After a measurable time lag, labelled RNA begins to accumulate in the cytoplasm. These experiments have been interpreted as a demonstration of RNA synthesis in the nucleus and the transfer of this molecule to the cytoplasm. With this explanation it has been implied or stated that the nucleus is a principal site of RNA synthesis. The early incorporation of activity into the nucleus certainly does not appear open to any other interpretation than, at the very least, a rapid synthesis of RNA in that location.

The three types of experiments were:

1. A time-study of RNA synthesis in the nucleus and cytoplasm with [^3H]-cytidine continuously present in the medium.
2. A study of the pattern of labelling in the nucleus and cytoplasm after a short exposure to [^3H]-cytidine.
3. Investigation of the capacity of nucleated and enucleated cells to incorporate [^3H]-cytidine into RNA.

All three experiments lead to one general conclusion; all RNA is synthesized in the nucleus, and cytoplasmic RNA is of nuclear origin.

Figure 1 shows the pattern of [^3H]-cytidine accumulation into nuclear and cytoplasmic RNA of *Tetrahymena*. In the first group of experiments, 10 $\mu\text{c.}$ ml. of [^3H]-cytidine were added to an early log phase culture. At intervals of a few minutes, groups of cells were withdrawn, dried on slides,

* Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

fixed, extracted to remove acid-soluble material, and autoradiographed. Within only 1.5 min. after addition to the medium, [^3H]-cytidine is taken up, converted to the appropriate form and incorporated into nuclear RNA (Fig. 2(a)). After 5 min. the rate of [^3H]-cytidine incorporation into the nuclear RNA rises. Incorporated activity is not detected in the cytoplasm until about 12 min.; in contrast, the nucleus is densely labelled (Fig. 2(b)). After 12 min., label accumulates steadily in cytoplasmic RNA but the rate of accumulation of radioactivity in the nucleus recedes to a lower value at about 25 or 30 min. At 35 min. the nucleus and cytoplasm are equally labelled (Fig. 2(c)), and at 60 min. the cytoplasm contains more than twice as much label as the nucleus. The nucleus at this time, however, is still

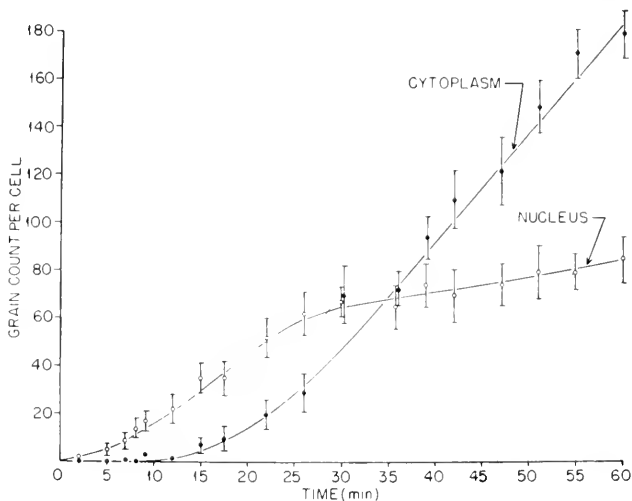


FIG. 1. The two curves show the time course of the total amount of [^3H]-cytidine incorporated into RNA of the nucleus and cytoplasm of *Tetrahymena* with the isotope continuously present in the medium. Each point is the mean grain count for autoradiographs for 23 to 26 cells. The range for each point indicates 95% confidence limits.

more densely labelled. Ribonuclease digestion shows that DNA synthesis contributes very little to this incorporation. Unlabelled deoxycytidine has been added to the medium with the intention of minimizing [^3H]-cytidine entrance into DNA in all experiments. The time of appearance of tritium in cytoplasmic RNA varies from one experiment to another. In one case it occurred slightly earlier than 13 min. and in another experiment did not begin until 25 min. In the latter experiment, the longer delay is probably related to a short interruption in cell proliferation imposed by transfer of the log phase cells to fresh nutrient medium just before the experiment was begun.

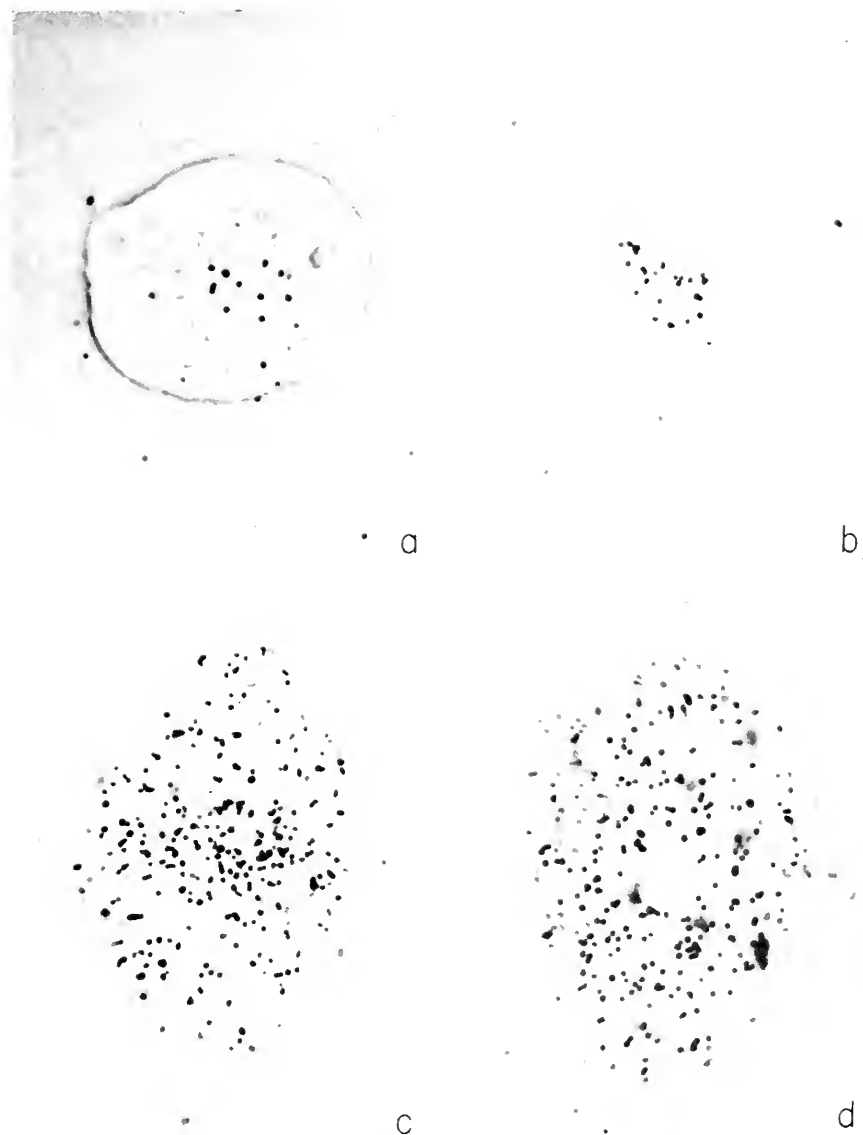


FIG. 2. (a) Autoradiograph of a *Tetrahymena* incubated in [^3H]-cytidine for 1.5 min. All incorporation is localized in the nucleus.

(b) Autoradiograph of a *Tetrahymena* incubated in [^3H]-cytidine for 12 min. All label is still localized in the nucleus.

(c) Autoradiograph of a *Tetrahymena* incubated in [^3H]-cytidine for 35 min. Nucleus and cytoplasm contain equal amounts of label, although the nuclear label is still more dense.

(d) Autoradiograph of a *Tetrahymena* incubated in [^3H]-cytidine for 12 min, followed by incubation in non-radioactive medium for 88 min. The cytoplasmic RNA is heavily labelled, but the nucleus contains no labelled RNA.

The delay in the appearance of cytoplasmic labelling in each experiment could conceivably be explained in one other way besides the hypothesized transfer of RNA from the nucleus to the cytoplasm. The nucleus might be the exclusive site of some contribution to RNA synthesis which precedes polymerization, i.e. some step in the conversion of nucleoside to nucleoside triphosphate. According to this hypothesis, the delay in cytoplasmic labelling might be considered as a measure of time for the labelled triphosphate to be formed in the nucleus and delivered to points of RNA synthesis in the cytoplasm. No evidence has been found in the literature that any such activities are localized in the nucleus.

These results with *Tetrahymena* are interpreted as evidence that RNA moves continuously from nucleus to cytoplasm. In view of the very rapid arrival of [^3H]-cytidine in the nucleus, it seems unlikely that the relatively long lag in the appearance of RNA bound tritium in the cytoplasm could result from cytoplasmic RNA synthesis being delayed until some cytidine-derived precursor of RNA could first diffuse out of the nucleus.

Initially the nucleus curve for [^3H]-cytidine incorporation shows a short lag, which probably reflects the time required for [^3H]-cytidine or a cytidine derivative to be built up in a precursor pool. The slope of the nuclear curve subsequent to the lag does not represent the rate of RNA synthesis in the nucleus but is a composite of rates of several events. During the entire course of the curve the average rate of increase of radioactivity in the nucleus is decreased by cell division, which occurs continuously during the experiment. At each cell division, the activity of the nucleus is divided between the two daughter nuclei, thus lowering the average amount of activity per nucleus. For most of its course the slope of the curve is also decreased by the shift of radioactivity from the nucleus to cytoplasm. The slope of the cytoplasmic curve is also decreased by cell division and possibly by some breakdown of RNA, although the occurrence of the latter seems doubtful. Granting that RNA does move from nucleus to cytoplasm, the lag in the cytoplasmic curve also suggests that there is a delay between the fixation of [^3H]-cytidine into an acid-insoluble polymer, very probably RNA, and the transfer of the completed RNA-protein molecule into the cytoplasm.

A number of studies [4-7] have shown that incorporation of radioactivity into nuclear RNA during a brief exposure to label is observed to disappear from the nucleus with concomitant appearance of labelled RNA in the cytoplasm when the cells are subsequently transferred to and incubated in a medium containing no label.

In the second group of experiments, *Tetrahymena* were exposed to a pulse of [^3H]-cytidine, and the distribution of labelled RNA followed after removal of exogenous [^3H]-cytidine. Figure 3 describes the results of the experiment. Ten $\mu\text{c./ml.}$ of [^3H]-cytidine were added at time zero. The

Tetrahymena were centrifuged out of the radioactive medium and re-suspended in medium containing unlabelled cytidine at the same concentration. This first washing resulted in a twenty-five-fold dilution of the [^3H]-cytidine and was completed at 12 min. At 12 min. all incorporated cytidine is still localized in the nucleus (Fig. 2(b)). The washing procedure was repeated three more times to give a 150 000-fold dilution of the isotope by 30 min.

After the first washing the incorporation of radioactivity into nuclear RNA continues for another 40 min., indicating a large pool of [^3H]-cytidine or its derivatives that could not be washed out of the living *Tetrahymena* with medium containing unlabelled cytidine. Cytoplasmic label begins to appear at about 15 min. Forty minutes after the first washing

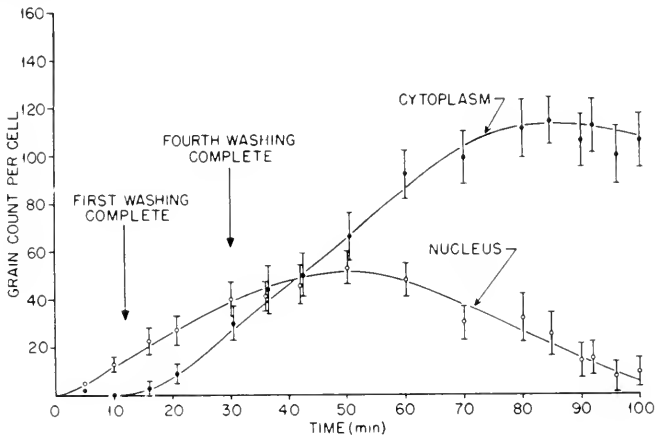


FIG. 3. The two curves describe the total amount of incorporation of [^3H]-cytidine into RNA of the nucleus and cytoplasm. At 12 min. the cells were washed free of the isotope with non-labelled medium. The range for each point indicates 95% confidence limits.

the density of label in the nucleus begins to decrease and by 100 min. has fallen to about 10% of the peak level of 50 min. Only a small fraction of this decrease can be ascribed to dilution through cell division. During this decrease the cytoplasmic label per cell increases until the rate of cytoplasmic labelling per cell equals the rate of dilution by cell division. This transient balance occurs at about 80 min. In the last 20 min. of the experiment, cytoplasmic label per cell is decreased more rapidly by cell division than it is built up by newly labelled RNA. At 100 min. approximately one-third of the nuclei contain no radioactivity (Fig. 2(d)). The remaining two-thirds of nuclei contain small amounts of activity, about half of which is RNase removable. The remaining trace of activity is presumed to be incorporated into DNA.



FIG. 4. An enucleated and a nucleated fragment exposed to [^3H]-cytidine for 2 hr. The RNA of the nucleated fragment is heavily labelled. The enucleated fragment contains no incorporated label.

In this pulse experiment the densely labelled nucleus in an unlabelled cytoplasm found at 12 min. contrasts sharply with unlabelled nucleus surrounded by heavily labelled cytoplasm at 100 min. (Figs. 2(b) and 2(d)). The presence of an unlabelled nucleus surrounded by heavily labelled cytoplasm not only indicates a transfer of RNA from nucleus to cytoplasm but suggests in addition that the transfer of RNA in the reverse direction, from cytoplasm to nucleus, does not take place. This distribution of labelling also implies that if breakdown of cytoplasmic RNA does occur, the products are not used by the nucleus for RNA synthesis. Similar conclusions were suggested by nuclear transplantation studies in amoeba by Goldstein and Plaut [8].

The experiment in Fig. 3 also demonstrates that the pool into which [^3H]-cytidine (or its derivatives) enters must be large since tritium becomes incorporated into RNA long after [^3H]-cytidine has been eliminated from the medium. Because the pool cannot be washed out of living *Tetrahymena* with non-labelled medium, it may be that the [^3H]-cytidine has been converted to a form which is bound (but still acid-soluble) or which does not readily pass through the cell membrane. The pool may be in the form of mono-, di-, or triphosphates of cytidine. Whether the pool is localized in the nucleus or cytoplasm or is present in both places is not known.

By removing the nucleus from a cell, it becomes possible to compare the capacities of nucleated and enucleated cells to synthesize RNA. *Tetrahymena* were cut into nucleated and enucleated fragments with a glass needle controlled by a micromanipulator. All of the nucleated fragments survive longer than 40 hr. and most of them regenerate and resume proliferation. The enucleated fragments survive in the complete nutrient medium for 10 to 40 hr. and move about by ciliary activity.

Enucleated and nucleated fragments of *Tetrahymena* have been incubated for 20 to 240 min. immediately after cutting, in complete medium containing [^3H]-cytidine. The incorporation of activity into nucleated fragments is always intense (Fig. 4). With short incubation (up to 90 min.) the nucleus is more densely labelled than the cytoplasm. After that time, labelling is so heavy in both nucleus and cytoplasm that no difference between the two sites is apparent. About thirty-five enucleated fragments of cytoplasm have been studied, and none has been found to incorporate [^3H]-cytidine into RNA (Fig. 4).

In sharp contrast, enucleated *Tetrahymena* are still capable of incorporation of [^{14}C]-amino acids. This activity occurs at a lower rate than in the nucleated fragments of comparable size. The [^{14}C]-amino acids are presumably incorporated into protein since they are not removed by 10 min. extraction with 5% TCA at 90° and ether-alcohol treatment for 10 min. This capacity of enucleated *Tetrahymena* to incorporate [^{14}C]-amino acids drops very rapidly after enucleation. By 6 hr. the incorporation

ceases completely, although the enucleated pieces remain motile long beyond this time.

The cutting experiments show that the cytoplasm is completely incapable of incorporating [^3H]-cytidine into RNA when the nucleus is absent from the system. As in other enucleated cell types cytoplasmic incorporation of amino acids continues. These enucleation experiments with *Tetrahymena*, combined with the time-labelling study (Fig. 1) and the pulse experiment (Fig. 3) substantiate the thesis that all RNA is synthesized in the nucleus and that nuclear RNA is the source of cytoplasmic RNA. The fate of "transfer RNA" (soluble RNA) in all of these experiments is not entirely certain; it is possible that some or all of this fraction might have been lost during the extraction procedures prior to autoradiography. For this reason, cytoplasmic labelling of transfer RNA with [^3H]-cytidine might, therefore, have escaped detection.

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Discussion

DAVIS: Your curve showed considerable increase in total counts in the cell if you add together the nucleus and cytoplasm after division has started. How can you account for the source of all that further radioactivity?

PRESCOTT: We can account for this because the cells contain a pool of cytidine and its derivatives that cannot be washed out of living cells. Dr. Bruce Jacobson and I have started some studies on this pool in *Tetrahymena* and we already know that it is in fact very large. We haven't really identified it yet but suspect that it is composed of CMP, CDP, and CTP.

DAVIS: I missed the point of technique; didn't your method of autoradiography involve getting rid of that pool first?

PRESCOTT: Yes, I am sorry that I didn't make that clear. The cells were prepared for autoradiography in such a way as to remove all acid-soluble material and to give a picture of incorporation only.

CHARGAFF: Does *Tetrahymena* contain cytidine deaminase?

PRESCOTT: I don't know.

CHARGAFF: Because if you rely on grain counts you do not know that it is still cytosine derivatives that you are still following.

PRESCOTT: True. But we can say that this incorporated radioactivity is RNA-ase sensitive, i.e. that it is 96-98% removable with RNA-ase treatment.

CHARGAFF: In other words it could not have gone through a cyclic process by which deamination could give a uridine derivative which was then methylated and so on.

PRESCOTT: And got into DNA?

CHARGAFF: Yes, there are some indications that ribosides can go into deoxy-ribosides; we heard about that from Dr. Reichard the other day.

PRESCOTT: We checked this point and found an appreciable amount of cytidine going over into DNA. We next added unlabelled deoxycytidine to the medium, and empirically it proved to be a good preventative for cytidine getting over into DNA.

SIEKEVITZ: When you did the washing experiment and the activity of the RNA in the nucleus went up, you made the assumption that you were not washing out the precursor pool of radioactivity. You could make the same assumption about the cytoplasmic RNA, that the washing experiment was not washing out any radioactivity from the pool there. This would go against the idea of nuclear RNA coming out into the cytoplasm.

PRESCOTT: Except that there is such a long lag between the presentation of radioactive cytidine to the cell and the appearance of labelled RNA in the cytoplasm. You are quite right about this in one respect, however. One possible interpretation is that the cytoplasm doesn't have the necessary kinases but the nucleus does and that cytidine passes through the cytoplasm into the nucleus where it is transformed into CTP. This in turn might leak into the cytoplasm where it would be used for cytoplasmic RNA synthesis. I personally don't believe that this happens, but it is one reason why we are looking into the pool question, particularly whether the cytoplasm has the capacity to convert cytidine into CTP.

HERBERT: I was wondering about the resolution of your method. Is there not the possibility that there is a sizeable pool of soluble RNA in the cell into which radioactivity is going which you can't resolve by your technique?

PRESCOTT: We are a little uncertain about the fate of soluble, or transfer, RNA in these experiments. We have had some help from Dr. Waldo Cohn and hope to settle the question. There could be terminal labelling in transfer RNA, and then loss of the label during the acid-extraction prior to autoradiography. Approximately 5% of the RNA in this cell is probably of the soluble type. We really don't know for sure what is happening to that RNA. We hope to decide whether soluble RNA stays in the cell with our treatment by labelling it with tritiated pseudo-uridine. In this connection we are also interested in the question of where the soluble RNA is synthesized; in the nucleus, cytoplasm or both places?

HERBERT: If it were floating free or soluble in the cytoplasm and not concentrated as it is in particles in the nucleus, then it would be very difficult to settle this. Is that not true?

PRESCOTT: We would pick it up. These cells are fixed in a manner which we believe precipitates the soluble RNA, but we are not sure that we are not losing some.

ALLFREY: I would like to raise a point that one must make the distinction between end-group labelling of RNA and net synthesis of RNA as I am sure

Dr. Prescott knows; but there is another problem which arises in experiments of this sort and that has to do with your observed lag period, and that may involve the fact that the concentration of precursor in the nucleus soon exceeds that in the medium and far exceeds that in the cytoplasm, so you get an apparent synthesis first in the nucleus.

PRESCOTT: We are aware of this possibility. We just simply prefer a more positive conclusion. I am willing to consider these criticisms seriously. We also are faced with the possibility that RNA synthesis may take place in the nucleus and this RNA is rapidly broken down; the breakdown products might leak into the cytoplasm and only these might be used for cytoplasmic RNA synthesis. There are a number of other alternatives, but I think that the interpretation I have made of these data are more probably correct; they also require the least number of additional assumptions.

ALLFREY: If I had your results I would draw the same conclusions.

Cell Division and Protein Synthesis

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I. The synchronized *Tetrahymena* system

In the course of the 7 years since Hotchkiss [4] for bacteria and we [10, 14] for a protozoon organism (*Tetrahymena pyriformis*) proposed temperature changes as a tool for phasing or synchronizing cell populations, this field has been rapidly expanding.

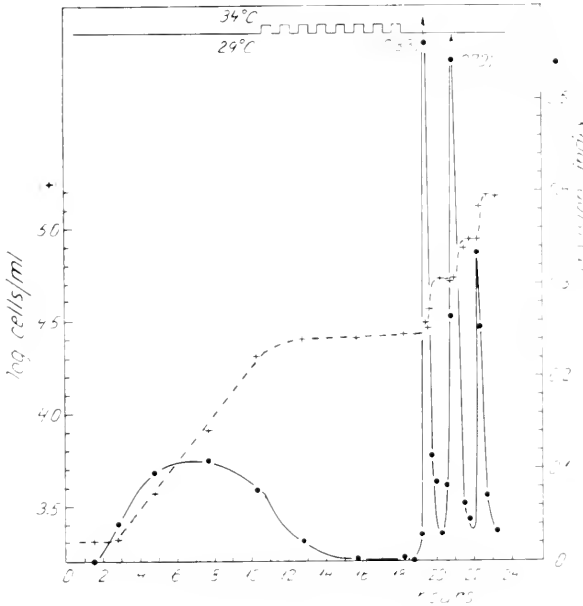


FIG. 1. The continuous curve represents division index, and the broken curve represents cell counts. (From Zeuthen and Scherbaum [14]).

The synchronous *Tetrahymena* system is demonstrated in Figs. 1 and 2. In Fig. 1 the stippled curve represents the logarithms of the cell counts per ml. During the first 11 hr. growth is at 29° C. which is optimum (28–29° C.). We observe first a lag-phase, then a log-phase of growth. During

the subsequent 7½ hr. the temperature is shifted eight times, and in a regular manner, between 29° C. and 34° C. This blocks cell multiplication. The lower curve (division index = cytokinetic cells/total cells) shows that in response to the temperature changes cells in division complete this process while no new cells enter into division. At subsequent constant 29° C. almost all cells divide at the same time, but only after a delay of 1½ hr. The cells complete this division together and they enter a second and a third division at 2-hr. intervals. This is reflected both in the population counts and in the division indices. In Fig. 2 the fully-drawn curve repeats and extends the broken curve from Fig. 1. Only the counts are

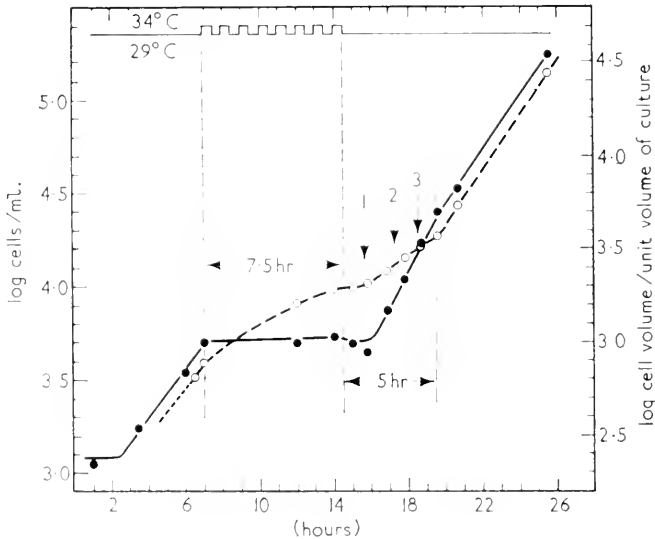


FIG. 2. The continuous curve represents cell counts, the broken curve represents volume of cells per unit volume of culture. (From Zeuthen and Scherbaum [14]).

not spaced close enough to describe the synchronous division steps. In Fig. 2 the broken curve is for total cell volume per aliquot. During the phase of shifting temperature the average cell grows to about three times its average logarithmic size. This is reversed during the synchronous division steps which occupy about 5 hr. as indicated. The relative position and the slopes of the two curves suggest complete reversibility of the induced division synchrony. Cells which through synchronous division have reverted to logarithmic growth may be resynchronized by the method shown in Figs. 1 and 2. The cells which after the heat shocks make ready for their first synchronous division are abnormally large, not only by volume but also by dry matter, protein content, RNA, DNA and by nuclear volume [2, 1, 7, 14, 12 (review)]. All measures increase more or

less in parallel during the period of blocked cell division, and at least by more than a factor of 2. The base ratios in both nucleic acids remain constant [7].

2. Studies with base analogues

While rather extended biochemical studies of the *Tetrahymena* system have yielded much valuable information, they have not greatly helped us to understand the biochemical mechanisms by which temperature changes induce the division synchrony. Figure 1 shows that no cells divide during the first hour which at constant 29° C. follows the period of changing temperature. This observation, extended in careful studies by Thormar [11], shows that recovery from temperature damage must take place before the cells divide. The damage brings all cells into a common situation with respect to their preparation for subsequent division. We have studied the recovery by the use of antimetabolites of various sorts. The cells are synchronized in a 2% proteose-peptone medium, fortified with 0.1% (more recently with 0.4%) liver extract L (Wilson Labs.). In some experiments we applied an extra period of elevated temperature (an extra "temperature shock"). Before the shock the organic medium was replaced with a simple inorganic medium [2]. The cells divide in standard time after the last shock, whether this is applied in the proteose-peptone or in the inorganic medium. Cells in the latter medium shall be referred to as "washed cells".

The time of maximum engagement in divisions 1, 2, and 3 (proteose-peptone medium) and in division 1, often 2 ("washed cells") can be determined with great accuracy. Consequently, it is possible to quantize the division-delaying effect of an antimetabolite which is added at a defined time before division. The changes in the division index is followed in up to twenty dishes each of which holds 1 ml. of the population. Frequent visual inspection of each dish is made at noted times. The percentage of cells in fission is quickly estimated. Curves through the estimates permit that we fix accurately ($\pm < 3$ min.) the time when in a dish a maximum of cells show fission. Counting is not necessary for sound estimates to be made. As a check of the method we have established that the results of two or more independent observers agree nicely. The effect of an antimetabolite is given by the delay of division relative to the proper control. Because many dishes are followed drug concentrations and other factors are easily varied in parallel runs.

Out of a considerable number of purine- and pyrimidine-analogues tested [13] only 8-azaguanine and 6-methylpurine were found to inhibit the first synchronous division in the washed cells. Furthermore, the two analogues were inhibitors of this division only when added before the lapse of about half the time which the controls require to prepare division

at constant 28° C. 8-Azaguanine is nicely antagonized by guanine, guanosine, adenine and adenosine. 6-Methylpurine is antagonized by adenine and by adenosine, not by guanine.

The general picture invites the suggestion that base analogues interfere much less readily with the cell's preparation for synchronous division at the level of synthesis of the two nucleic acids than at the level of the co-factors (GTP and ATP) involved in protein synthesis. This leads to the hypothesis [13] that the temperature-shocked cells, while overcharged with nucleic acids and proteins, are short of one or several proteins which are specifically related to the process of cell division. According to the hypothesis this situation is corrected by new synthesis after the termination of the temperature shocks.

3. Studies on DNA

In view of suggestions made by Scherbaum [8] it is recalled that in early work with Dr. E. Hoff-Jorsensen we [14] found close to constancy of the ratio DNA/unit cell volume. As shown in Fig. 3 this is for the period of shifting temperature when the average cell increases by a factor 2·5–3 and for the period of synchronous divisions during which the average cell size regulates to normal. This work gave no indication of a special role played by DNA in the induction by heat of the division synchrony.

As a continuation of this work Dr. Rose Cerroni in our laboratory independently made an observation also reported by Scherbaum [8]. Tritiated thymidine (specific activity 1·9 c./mM, 5 μ c. per ml. cell suspension, henceforth to be referred to as the standard dose) added to populations of 50 000–100 000 cells/ml. label the nucleus of only a fraction of all cells. We used the labelled compound undiluted with cold thymidine externally added. The total amount of thymidine represented by the standard dose is of the order of only 1/10 of the amount of DNA in all cells in the sample.

Whether logarithmic cells are studied in proteose-peptone (0·4 liver extract) or whether they are transferred to the inorganic medium before the addition of the labelled thymidine only 27–37% of all nuclei take the label. These experiments suggest that the radioactive compound is soon removed by the one-third to one-half of the cells in the randomized population which can be expected (cf. [5] and [6]) to synthesize DNA at any one time. This view is further supported by the observation that all (98%) nuclei become labelled if three additional standard doses of tritiated thymidine are given to the same cells for 4 min. at 50 min. intervals.

A standard dose of tritiated thymidine offered to the synchronized cells in proteose-peptone again label only a fraction of the nuclei; 20% when

offered at time EH,* regularly dropping percentages when the compound is added later, finally almost no labelling when cells in maximal division 1 are exposed. This can be repeated with "washed cells" (defined in Section 2). Immersion of washed synchronized cells into a solution of 5-fluoro-2-deoxyuridine (5×10^{-3} M) at EH, thus prior to the addition of the tritiated thymidine reduces to 6-12% the average number of tracks over the nucleus which takes the label. It does not significantly alter the percentage of labelled cells. This is so for all time points studied. The long exposures to this base analogue interfere neither with division 1 nor with

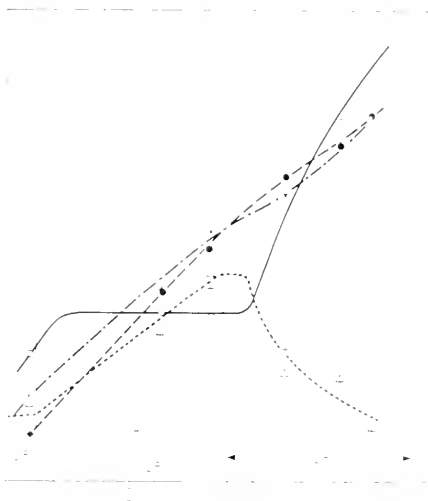


FIG. 3. The open circles represent log cell number per ml. culture. The filled circles represent DNA (microbiological assays) and the crosses (\times) packed cell volume per ml. culture. Increases in cell volume and in DNA go parallel, for the culture and for the average cell. Plotted from Table 1 in Zeuthen and Scherbaum [14].

division 2. It is not yet proven, but it can be suggested that two synchronous divisions can be developed without *de novo* synthesis of DNA after the termination of the temperature shocks.

4. Studies with amino acid analogues

The ideas developed in Section 2 have stood the first test with amino acid analogues. The experiments were performed in association with cand. mag. Leif Rasmussen.

DL-*p*-fluorophenylalanine (*p*-F-Phe) is a strong inhibitor of cell division

* The time when the last temperature shock ends, as shown by a signal on the control watch.

in *Tetrahymena* cells, synchronized as well as normal cells from logarithmic cultures. *p*-FPhe is antagonized competitively by phenylalanine. In principle we have found no difference in response between synchronized cells and cells from a logarithmic population. The response of the former cells is only much more easily analyzed than that of the latter, so our work on logarithmic *Tetrahymena* cells has mostly served as a control on results obtained with the synchronized cells.

Figure 4 shows two combined experiments for cells which have been synchronized and remain in proteose-peptone (0.4% liver fraction). The two upper curves show the three first synchronous divisions (1, 2, and 3)

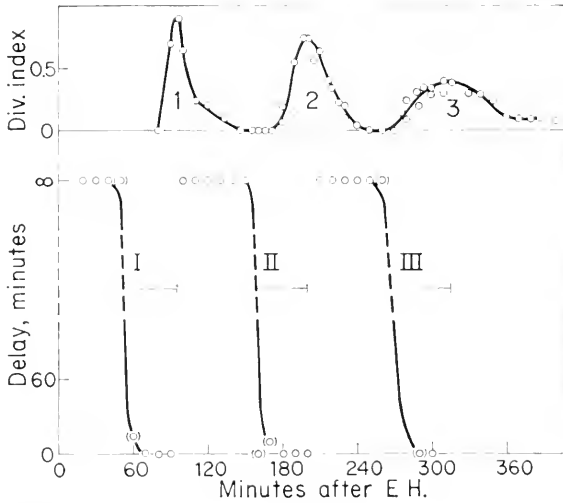


FIG. 4. *Upper curve*: Division maxima 1-3 in control represented by the changes in time of the division index. *Lower curves*: Delays of divisions 1-3 (curves I-III) as a function of the time of immersion of the cells into *p*-FPhe, 16 mM in proteose-peptone.

in the main controls. Divisions appear as maxima on the curves for the division index. The inhibitor is 16 mM *p*-FPhe. It is added to the cells for continuous exposure at the times (abscissa) indicated by the position of the points on curves I, II and III. The ordinate of a point represents the delay (relative to the parallel control) of division 1 (curve I), division 2 (curve II), and division 3 (curve III). The infinity sign indicates block of the subsequent division. All observations are at 28° C.

Obviously, there is a critical time before a division when a decision is made whether or not that division can be blocked by the amino acid analogue. This time (interpreted as shown in Fig. 4) is 42 mins. before division 1, 40 min. before division 2, and 47 min. before division 3. We

have found it to be around 60 min. before division in the logarithmic cell growing in proteose-peptone plus 0.4% liver extract.

We have further analyzed this effect by exposing the cells (medium as before) to 16 mM *p*-FPhe for only 20 min. After that time the inhibitor is removed by three washings with fresh growth medium, using the hand centrifuge. The control is similarly treated in a parallel tube. The results are shown in Fig. 5. In this case we have only delay, never block of division. The delays are plotted against the time of immersion into the inhibitor. Curve I represents the delay of division 1 (lengthening of time interval from EH to division 1). Curve II measures the delay of division 2 in terms of extended intervals between divisions 1 and 2. Several experiments are combined but little attempt has been made of keeping them apart because

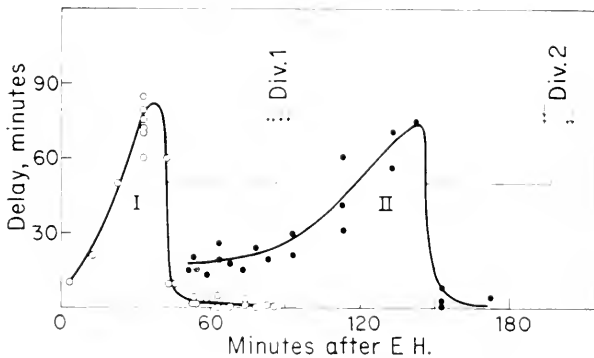


FIG. 5. Delay of synchronous division in proteose-peptone by 16 mM *p*-FPhe for 20 min. The abscissa is the time when the exposure is initiated. *Curve I*: Delay of division 1. *Curve II*: Delay of division 2 (lengthening of time interval between divisions 1 and 2).

both the synchronizations and the responses to the analogue are so nicely reproducible. Only the arrows which show the times for maximal division 1 and 2 separate between experiments. The results confirm those of Fig. 4 in showing that there is a critical time about 44 min. before division 1, and 50 min. before division 2 when the response to *p*-FPhe drops sharply. The new information conveyed by Fig. 5 is that the reaction to a standard treatment with the analogue increases (curve I) from EH to reach a maximum value (at 35 min.) just before the drop in advance of division 1. Further (curve II), that this cyclic variation repeats itself between divisions 1 and 2 and even extends back in time (left part of curve II) to before division 1. Thus, 20 min. of exposure to the amino acid analogue, made before division 1 delays the preparation, not of this immediate division, but of the next one. However, this delay of division 2 is only slight and of the same order as the exposure time (20 min.) to the analogue. Curve I

shows equally short delays of division 1 when exposure is immediately after EH. However, for many time points in the cell's cycle the delays of division are much longer than the time for which the cells were in contact with *p*-FPhe.

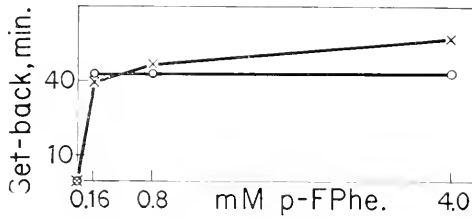


FIG. 6. "Set-back" against concentration of *p*-FPhe in inorganic medium. The exposure is always for 20 min. *Crosses*: Division 1—Cells immersed at 25 min. after EH. *Circles*: Division 2—Cells immersed at 55 min. after EH.

A complicating factor in the experiments described is in the complexity of the growth medium. For cells ("washed cells") in the inorganic medium the extracellular pool of amino acids is nil and the sensitivity of the cells to amino acid analogues is much increased. For the washed cells we have

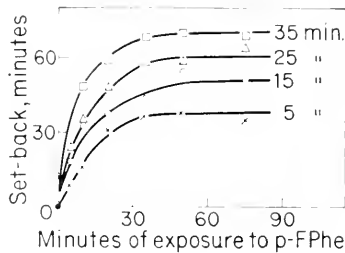


FIG. 7. "Set-back" (ordinate) as a function of the duration (abscissa) for which exposure is made to 0.8 mM *p*-FPhe in inorganic medium. The curves are for different times after EH of beginning treatment with *p*-FPhe. EH is the time when the last temperature shock ends.

been able to define combinations of concentrations (Fig. 6) of *p*-FPhe and of exposure times (Fig. 7) which give maximal delaying effects.

Figure 6 shows the relation between response of the washed cells and concentration of *p*-FPhe added at 25 (crosses) and 55 (circles) min. after EH. Exposure is for a standard time of 20 min. The analogue is removed by four washings with the inorganic medium. Washing is only complete if 1 mM DL-phenylalanine is added. So this was done. The response is represented by the "set-back", which is equal to the delay of the division, minus the time for which the cells were in contact with the inhibitor. The crosses represent "set-backs" for division 1, the circles relate similarly to division 2. For both divisions maximal effects are obtained with 0.16 mM

p-FPhe and with concentrations above this. We note once more (cf. Fig. 5) that when *p*-FPhe is added (at 55 min.) later than at a critical time before division 1 this division is not delayed. This is not because a permeability barrier to *p*-FPhe is established at this time. If it were, division 2 would not be delayed, which it is.

Figure 7 shows the relation between exposure time (abscissa) and "set-back" (delay of division, minus the varied time for which exposure to the drug is made) of synchronized, washed *Tetrahymena* cells dumped into the inhibitor (0.8 mM *p*-FPhe) at defined times after EH. Separate curves are shown for cells which are immersed at 5, 15, 25, and 35 min. after EH, thus at intervals of 10 min. The maximal set-back takes time to develop but it is nicely defined in each case. It becomes roughly 10 min. longer for every 10 min. by which we postpone the addition of the analogue.

From Fig. 6 we learned that almost the same effect is obtained whether exposure for 20 min. is to 0.16 or to 4.0 mM *p*-FPhe. We shall assume that a given intracellular level of *p*-FPhe is attained the earlier the higher is the external concentration of the analogue. Then, with 0.8 mM outside concentration the intracellular concentration of the analogue should reach a maximally inhibitory concentration in a fraction of the 20 min. for which (in Fig. 6) exposure is made. If this is so, then the curves of Fig. 7 do not measure penetration rates of *p*-FPhe. A different interpretation is based on the idea that *p*-FPhe penetrates the cells fast enough to produce a quick block for protein synthesis. The washed cells are starving cells, so protein synthesis must be from an amino acid pool which is supplied continually by catabolism working on cellular proteins. The method we use can trace the synthesis only of proteins which are related to division and which for this reason we shall call "division proteins". If also the "division proteins" show a turnover then they shall decay as soon as we block protein synthesis with *p*-FPhe. Indeed, the curves of Fig. 7 may largely represent decay of "division proteins" piled before the addition of *p*-FPhe.

Figure 8 is based on the data presented in Fig. 7. It relates "set-back" and time of the beginning of the exposure to the analogue (0.8 mM *p*-FPhe). Separate curves are given for the six exposure times of 5, 10, 20, 35, 50, and 75 min. All curves tend to be linear so that intersection points with the time axis at -6, -11, -27, -29, -29, and -30 min. can be defined. The slope is close to 45°. Exposure for 35 min. and more gives maximal set-backs (Fig. 7). In the light of our "block-and-decay" hypothesis, Fig. 8 indicates that a developing store of "division protein" decays fully at any time when *p*-FPhe is added to stay for 35 min. or more. Irrespective of when added, when the analogue is again removed, the cells are empty of "division protein". As a consequence the treated cells have a standard time to go before they will divide. This time is 110 min. and equals the time which the controls take to go from EH to division 1 (80 min.) plus those

30 min. which is represented by the distance from EH to the most left intersection point in Fig. 8. This figure also suggests that by the time EH the synchronized cells contain some "division protein", though not enough for a division. How much they contain we cannot say. Our graphs only compare times measured in two different ways. Linearity of a curve which slopes 45° means that time spent on synthesis before the addition of *p*-FPhe is fully lost at the time when the analogue is again removed.

Returning to the experiments recorded in Fig. 5 it is observed that both curves I and II ascend towards the right in a non-linear fashion. In those experiments cells in proteose-peptone were treated for 20 min. with 16 mM *p*-FPhe. This combination of growth medium, exposure time, and

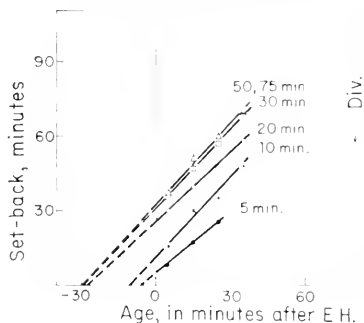


FIG. 8. "Set-back" against time since EH when the cells were immersed into 0.8 mM *p*-FPhe in inorganic medium. The separate curves represent various exposure times as indicated. The dashed parts of the curves are extrapolations. [Error in Figure: for 30 min., read 35 min.]

inhibitor concentration failed to give maximal set-backs as defined for the washed cells (Figs. 6 and 7). For this reason linearity of the rising limbs of the curves in Fig. 5 could not be expected.

Other amino acid analogues have been tested for comparison with *p*-FPhe. Ethionine, β -thienylalanine and canavanine seem to act in the same way as *p*-FPhe. So do chloramphenicol and puromycin, well-established inhibitors of protein synthesis. It is the results with the two antibiotics which have induced us to ignore the possibility of a significant synthesis of false proteins in the presence of an amino acid analogue. This possibility is under further study.

5. Conclusions

We have no evidence from the study of the synchronous cells that before every division *Tetrahymena must* produce RNA. With regard to DNA we have evidence that no new synthesis of this substance *needs* take

place after the termination of the last temperature shock and up to a time when two synchronous divisions have displayed themselves. This is perhaps not so strange since *Tetrahymena* is highly polyploid and probably becomes even more so when it is synchronized.

According to our interpretations *Tetrahymena* cells *must* produce "division proteins" before every division. This is so whether the cells are in the logarithmic growth phase, whether they are synchronized and grow in proteose-peptone, or, most importantly, whether they perform synchronous divisions without growth. During the period of cycling temperature used for the synchronization *Tetrahymena* grows large and produces a lot of proteins. However, it fails to produce "division proteins" to the level required for a single division. In fact we consider that the synchrony is induced because at EH all cells are equally low in "division proteins" which later, at constant 28° C., are produced synchronously to give rise to the first synchronous division in standard time.

For the synchronized cells in proteose-peptone increases in respiration, in dry matter, and in protein synthesis are discontinuous (review by Zeuthen [13]). In all these measures synthesis is slowed around division. The synthesis of "division proteins" is likely to be a small fraction of the total since it is observed also in starving cells. It may or it may not follow total synthesis and it may or may not be continuous through a series of divisions. However, that part of the synthesis which conditions a division seems to be sharply delimited and goes from 40–50 min. before one to 40–50 min. before the next synchronous fission. The way we interpret our results is that firstly the cell charges itself to a threshold level with "division protein". Then, around 40–50 min. before synchronous fission all "division protein" changes from a state in which it will decay in the presence of *p*-FPhe, to a state when it will not. So we think that stabilization has suddenly taken place. And this stabilization would be a condition for all the kinetic phases of the division process later to take place.

The drop before division 1 from maximum to no capacity to become set-back by *p*-FPhe occurs at the time when the anarchic field [3, 9] is just beginning to organize into the definitive oral membranelles of the second mouth. This is personal information from Dr. Joseph Frankel obtained under identical conditions in our laboratory.

This new orientation in space of previously synthesized kinetosomes may be one of the earliest manifestations of the action of the stabilized "division protein".

The work reported will be published in the *C. R. Lab. Carlsberg* by Rose E. Cerroni, Leif Rasmussen and the author.

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Discussion

MAZIA: One point made by Zeuthen could be stressed because it would apply to division in cells other than *Tetrahymena*. While the "division protein" may be crucial, it needs not represent very much protein. His proof is that the absence of availability of external nutritional sources for proteins makes little or no difference to the division of his synchronized cell populations. It has also been observed in studies on fission yeasts by Faed in Mitchison's laboratory that the cells may go through a complete division cycle or two in the absence of external nitrogen sources, producing small progeny. The "division protein" may be available in small but adequate amounts, may be supplied by conversion of other proteins, or may be made from the amino acid pool. The fact that it is crucial does not imply that it represents a quantitatively important fraction of the protein synthesis taking place during the growth-division cycle.

DAVIS: Dr. Zeuthen, did I understand correctly that during this period of growth without division DNA and RNA continued to be synthesized at normal rates? I wonder if your problem might be a little analogous to one observed with bacteria, where under the influence of many inhibitory agents at border-line concentrations the cells continue to grow and become tremendously elongated, but do not divide. The limitation appears to be the completion of the septum which leads to division. Could yours be a problem where the limiting factor is cell membrane formation?

ZEUTHEN: We think we have put our fingers—very lightly—on a protein which is specifically engaged in division but, as Dr. Mazia said, may be only a very small part of the whole cell. What does this protein perform, where does it sit? We do not know. Our problem could be analogous to the situation mentioned by Dr. Davis. We have incubated the washed cells with labelled amino acids. In radioautographs the label seems to sit everywhere. We may now try to fractionate for cell walls ("pellicles"), nuclei, particles and so on, to see if the label is attached predominantly to one of these organelles. Another possible approach would be that we try to separate the proteins after previous incubation of the cells for short and different times with labelled amino acids. One, or more, proteins might take the label in excessive amounts.

Structure and Function in Amoeboid Movement*

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Amoeboid movement is a process with which every biologist and student of biology is familiar. Yet, despite its fundamental importance, this form of cellular motility has been one of the most poorly understood phenomena in cellular biology. Nearly every generation of biologists over the last century and a quarter has produced a new explanatory theory, only to have it supplanted in the next generation by a totally different one. Theories of amoeboid movement seem now to have passed through a complete cycle, for the front- and tail-contraction theories, which I shall discuss here, both represent a return to the general idea, first expressed by Dujardin [9], that amoeboid movement is basically a contractility phenomenon. If one accepts this idea, then obviously the most fundamental question is the location of active contraction (i.e. the "engine") in the moving cell.

The streaming endoplasm has in the past been excluded as a possible site for this "engine" because this region of the cell has been assumed to have the physical properties of a Newtonian sol [12, 14]. Since structureless fluids can neither develop nor transmit tension, this concept of endoplasmic consistency led inexorably to the tail contraction theory [10, 13], according to which the endoplasm is moved passively by a pressure gradient generated by an actively contracting ectoplasmic tube. The concept of the endoplasm as a structureless sol also excluded any consideration of an alternative mechanism such as will be proposed below.

Several recent developments have led us to propose such an alternative mechanism. First, it has been pointed out elsewhere [3, 4] that many of the behavioural aspects of amoeboid movement are incompatible with the tail contraction theory in its present form. Second, it has been shown that amoeba cytoplasm will continue to stream after it has been dissociated from the cell [1, 5]. This capability was neither predicted nor explained by the tail contraction theory.

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Third, a new concept of amoeba cytoplasmic structure has emerged from recent rheological studies of consistency differences in various parts of the moving cell [2, 3, 7]. In contrast to the traditional "sol-gel" concept of amoeba structure, it has been shown that the axial portion of the endoplasm (Mast's "plasmasol" [14]) possesses weak gel structure. Velocity profiles of endoplasmic streaming within the ectoplasmic tubes of narrow, cylindrical pseudopodia of *Chaos chaos* were found to be similar to those found for plug flow of a non-Newtonian fluid in a tube [7], and were also very similar to velocity profiles of cytoplasmic streaming in

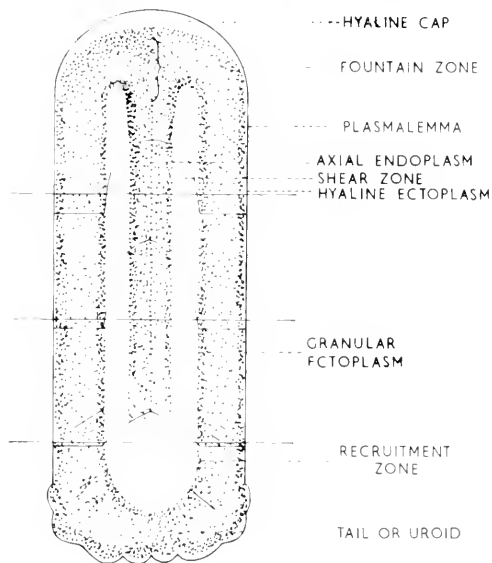


FIG. 1. A diagrammatic representation of the concept of amoeba pseudopodial structure discussed in the text. The superimposed curves are velocity profiles from the data of Allen and Roslansky [7].

myxomycete channels [13]. Plug flow occurs when the shear stress acting on a fluid near the centre of a stream is insufficient to cause significant rates of deformation (i.e. velocity gradients) in the fluid, but when the higher shear stresses near the walls exceed the yield point of the material (if it is a gel) its apparent viscosity is reduced to the range expected of true sols. The velocity profiles, by demonstrating the quasi-pseudoplastic nature of endoplasmic flow, have drawn attention to the presence of weak gel structure which might permit the development and transmission of tension. Only the tail endoplasm and the peripheral endoplasm of the "shear zone" (Fig. 1) have shown evidence of a low apparent viscosity. Studies with the centrifuge microscope have recently confirmed the

presence of this weak gel structure in the axial portion of the endoplasm. This region of the cell was found to offer visible resistance to the displacement of accelerated cytoplasmic inclusions [2]. These centrifugation experiments have confirmed the generally held concept of a more rigid consistency for the ectoplasmic tube as a whole (Mast's plasmagel [14]), but have revealed an unexpected gradient of rigidity in both the ectoplasm and axial endoplasm from a high at the front of the cell to a low in the tail.

The results of these and other rheological experiments, which have been summarized elsewhere [3], are presented schematically in Fig. 1. It is this concept of amoeba cytoplasmic structure which led to the new front contraction theory. It is perhaps simplest to outline the theory first, and then point out some of the experimental evidence which supports it.

First let us assume that the endoplasm (especially the axial portion) is uncontracted or relaxed cytoplasm. According to the theory, a given portion of this material begins to contract just before it splits and becomes everted to form the continually advancing ectoplasmic tube. The contraction is completed by the time this material has become incorporated into

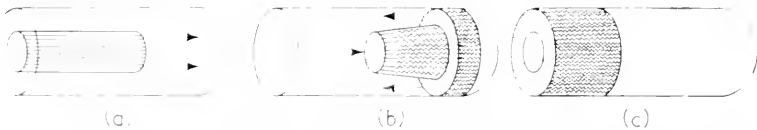


FIG. 2. A diagram to illustrate the fate of a cylindrical block of axial endoplasm as it contracts at the part of the cell as proposed by the fountain zone contraction theory [4].

the ectoplasmic region. During its passage through the region of the cell which we have termed the *fountain zone* (Fig. 1), this given portion of cytoplasm shortens (along the axis of the pseudopod) and thickens (radially) (Fig. 2). At the same time it develops tension, which is transmitted posteriorly through the axial endoplasm to "pump out" the tail. Increased cross-bonding during contraction in the fountain zone region causes localized syneresis, the fluid from which appears periodically in the hyaline cap. The increase in rigidity in the fountain zone accounts for the difference in consistency between the ectoplasmic tube and the endoplasm; this change is probably analogous to the increased rigidity which accompanies muscular contraction. The dilute fluid of the hyaline cap, which has been pressed out of the cytoplasm contracting in the fountain zone, is pumped tailward by the advance of the granular cytoplasm of the pseudopod within the loosely fitting plasmalemma. The hyaline ectoplasm serves as a channel between the plasmalemma and ectoplasmic tube through which the hyaline fluid travels to the tail region, where this fluid is returned eventually to the endoplasmic stream. The pull exerted on the axial endoplasm from the front may in part draw some of this fluid through the tail

ectoplasm so that it can be returned to the endoplasmic stream. If the contraction part of the contractility cycle involves syneresis, then this fluid must be resorbed in whatever part of the cell the relaxation part of the cycle occurs. It is also possible that some of the hyaline fluid is squeezed backward from the fountain zone as a counter-current. The theory further requires that the contraction remain localized in the fountain zone; hence, this front contraction theory has been named the "fountain zone contraction theory". In order to remain localized in the fountain zone, the contraction itself must be propagated posteriorly toward the axial endoplasm at approximately the velocity of forward endoplasmic displacement relative to the advancing tip.

The theory so far explains only endoplasmic displacement with respect to the ectoplasm. Locomotion can occur only if the ectoplasmic tube is attached at certain points, by means of the plasmalemma, to the substratum. The larger species of amoebae, such as *Amoeba proteus*, are attached near the middle of the cell at from one to several points [8]. If attachment does not take place, the ectoplasm and endoplasm are indeed displaced in opposite directions, a situation which has been called "fountain streaming" [3, 14].

The fact that we are now faced with two opposing contraction theories which postulate contractions localized at opposite poles of the cell makes it imperative to examine the question of exactly what constitutes evidence of an active contraction. In muscle, the measurement of tension developed or work performed removes all doubt as to whether an observed shortening is passive or active. In the amoeba, the situation is not so simple. The fundamental observational basis for the tail contraction theory is that the tail *shortens* [11]; many authors have uncritically accepted this as conclusive evidence for tail contraction. Actually, this shortening is *compatible with* the tail contraction hypothesis, but is also compatible with the hypothesis that the tail is "pumped out" as the new theory proposes. Even the measurement of tension between two points in the shortening tail would not settle the question, for some of the work done in the fountain zone contraction would appear as tension between two points in the shortening tail ectoplasm.

It seems reasonable to propose that only the development of tension or the production of large amounts of heat in a localized shortening region of cytoplasm should be considered conclusive evidence of an active contraction. Localized syneresis is probably also conclusive evidence of an active contraction, since syneresis is well known to result from increased cross-bonding in gels. Simultaneous shortening and thickening of a body of cytoplasm, however, is by itself not much more than suggestive evidence of active contraction unless it is accompanied by localized syneresis, tension development, or heat production.

The visible events in amoeboid movement are perfectly compatible with the fountain zone contraction theory but provide no conclusive evidence for it. On theoretical grounds it would be expected that a cylindrical block of endoplasm should widen in the fountain zone, and that in becoming ectoplasmic tube it should increase in cross-sectional area and shorten (Fig. 2). In fact, it does increase in cross-sectional area by a factor of 2 to 3 [15], depending on environmental conditions; a compensatory shortening also occurs [5]. The hyaline cap fluid, which is known to be produced by syneresis [3, 5, 6], appears only when there is forward flow of cytoplasm through the fountain zone. Hyaline caps erupt in different pseudopodia in the same cell at different frequencies, suggesting that different contractions in different pseudopodia are the sources of this fluid, rather than a contracting tail common to all of the pseudopodia. The existence of a fluid channel in the hyaline ectoplasmic region is shown by the fact that the plasmalemma is free to slide over the ectoplasmic tube in most parts of the pseudopod except at limited points of attachment [10]. Entry of the hyaline cap fluid into the tail region has not been demonstrated but can be perhaps inferred from the "softening" of tail ectoplasmic structure observed in the centrifuge microscope [2]. The development of tension in the axial endoplasm would be difficult to demonstrate directly by physical methods, but the development of tension would be perhaps the best simple explanation for the fact that birefringence is highest in the tail endoplasm, despite the modest velocity gradients developed there [3]. So far, none of these observations excludes the tail contraction theory completely.

The most direct evidence in support of the fountain zone contraction theory comes from a recent study of streaming in cytoplasm dissociated from the giant amoeba, *Chaos chaos*. About 6 years ago while working in Professor Runnström's laboratory at the Wenner-Grens Institute, I discovered that amoeba cytoplasm could continue to stream for periods of up to 1 hr. after it had been dissociated from the intact cell [1]. At that time, it was apparent that this phenomenon was neither predicted nor explained by the tail contraction theory, but there was then no alternative mechanism that offered an explanation. It seemed clear, however, that the cytoplasm possessed more structure than prevailing concepts allowed, and that streaming endoplasm might somehow be "self-propelled".

We have recently re-examined this phenomenon in the light of the concept of amoeba structure illustrated in Fig. 1 and in the light of the predictions offered by the fountain zone contraction theory [5]. Our data not only strengthen this concept of amoeba structure (Fig. 1) but also provide positive indications that the fountain zone contraction theory satisfactorily explains streaming in dissociated cytoplasm.

When the plasmalemma of an amoeba is ruptured while the amoeba

is held in a glass capillary, the cytoplasmic streaming organization of the cell often remains intact; the fountain streaming pattern continues with the capillary wall serving to replace the destroyed plasmalemma. When the fountain-streaming organization of the cytoplasm breaks down, it characteristically is replaced by one or more *loops* of streaming cytoplasmic material. In each streaming loop, cytoplasmic material moving toward the bend of the loop corresponds in origin and structure to the endoplasm of the intact cell; similarly, the material moving away from the bend corresponds to ectoplasm. The bend is thus a two-dimensional analogue of the fountain zone, which must have dissociated roughly into radial sections in order to form loops. There is a marked consistency difference between cytoplasm on the two arms of the loop, as can be deduced from the velocity

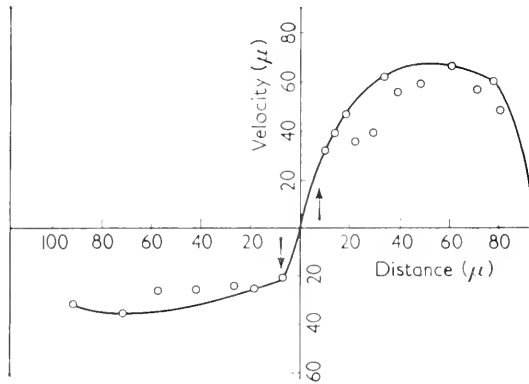


FIG. 3. A velocity profile across a loop of streaming cytoplasm dissociated from *Chaos chaos*. Note the difference in velocities (greater in the cytoplasm moving toward the head) and in the shape of the profile. Data of Allen, Cooledge and Hall [5].

profiles in Fig. 3. Therefore, the consistency change occurs at the bend. Cytoplasm moving toward the bend slows down, shortens (axially), thickens (radially), and gives up synergetic fluid which can be visualized as vacuole formation in the presence of traces of calcium ions. The observations listed so far are strongly suggestive of an active contraction at the bend of the loop. There is one point of evidence which appears to be conclusive: when cytoplasmic loops stream sporadically, a shortening can be seen to occur a brief moment before displacement of the endoplasmic and ectoplasmic arms of the loop toward and away from the bend. Thus the temporal sequence of mechanical events provides a seemingly unequivocal indication of an active contraction at the bend of the loops, and therefore probably in the fountain zone of the intact cell as well.

Some of the behavioural aspects of amoeboid movement which led to doubts concerning the correctness of the tail contraction theory appear to

be quite compatible with this front contraction theory. For example, it has been pointed out that occasionally the endoplasmic stream splits longitudinally, and portions of the stream move in opposite directions as if pulled by opposing fountain zones [2, 3]. The reversal of streaming also occurs as if most of the endoplasm were pulled instead of pushed in its new direction, for reversal begins first at the new advancing front and stops last at the old advancing front [3, 4]. Each front exhibits normal hyaline cap production cycles throughout the change. While it may be possible by means of additional assumptions to reconcile these facts with the tail contraction theory, it is important to realize that these observations are fulfillments of the predictions one would make from the fountain zone contraction theory even if one had never seen an amoeba.

The fountain zone contraction theory is only the first step toward the localization, identification, and understanding of the molecular mechanism of amoeboid movement. We can hope that the "engine" of amoeboid cells, once localized, will be easier to dissect and characterize by physical and chemical experiments. As has been pointed out elsewhere [3, 4], the principle behind the theory may have wider applications to other systems the mechanisms of which have been obscure, such as reticulopodial movement in foraminifera and certain cases of protoplasmic streaming in plants.

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Discussion

GOLDACRE: How would you account on your hypothesis for the fact that ATP injected into the cell causes a local contraction at the site of injection which then becomes the tail, not the front? The second question: I gather that your hypothesis requires a propagated contraction which is held in place by the U-shaped bend at

the end of your capillary tube. What happens when you blow the material out of your capillary tube on to a cover slip to form a circular drop? Do you then find a propagated contraction around the circle with no streaming?

ALLEN: In answer to your last question I think I would be restrained from carrying out such an experiment as you suggest, by the fact that the cytoplasm of the amoeba is so delicate that if one takes it out of the cell by anything but the most careful methods, it fails to stream. In answer to your first question I am aware of the ATP injection experiments which you reported in 1950, but I must remind you that you didn't give very much information about the time relations in these experiments or state the number of experiments that were performed. I should think it quite possible that by poking an amoeba with a needle or a pipette you might obtain almost any kind of behavioural result. I don't regard the changing of direction of an amoeba in one experiment in response to ATP injection as proof that phosphate energy from injected ATP has intervened in the contractile mechanism. There are too many unknowns. The results you reported are certainly compatible with this idea but it would take a great deal more data to prove it.

GOLDACRE: Many repeated experiments showed an immediate contraction at the site of injection of ATP. Injection of other substances (as we reported) had no effect. Ts'o and his colleagues reported, in 1956, similar results to ours for the microinjection of ATP into slime moulds; they also demonstrated a reversible lowering of viscosity of protein extracts from slime moulds each time that ATP was added to the solution; after the ATP was decomposed, the viscosity rose to its original value; this could be repeated indefinitely with the same protein solution, just as with actomyosin. Loewy in 1952 also reported this effect of ATP on slime mould extracts.

ALLEN: But again it has to be demonstrated that the energy from ATP has been utilized to stimulate the contractile mechanism; ATP may have many more possible effects than we now realize. The contraction you speak of is inferred from behaviour rather than directly observed.

Some Problems of Ciliary Structure and Ciliary Function

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The purpose of this paper is to review some of the problems of ciliary movement in the hope that new findings on ciliary and flagellar fine structure may shed light on the mechanisms responsible for the movement. Several questions are still to be answered:

1. By what mechanism do cilia and flagella work ?
2. In what respects does the fine structure of a flagellum differ from that of a cilium ? Is it possible to correlate such differences with their different modes of movement ?
3. What is the significance of the "magical $9 + 2$ filament arrangement" in cilia and flagella ? Are there meaningful variations in their arrangements ?
4. Is the ciliary beat (or the flagellar beat) to be regarded as a contraction process ? Are there significant similarities between these movements and the contraction of, for instance, a striated muscle ?

The observations that are presented here have been made with the electron microscope. The different types of cilia and flagella that have been chosen for study have only this in common: they have been subjected previously to a careful analysis with regard to their movements. The study has thus been intended to be an attack on the second question above. It was hoped that some definite conclusions could be made and that an answer to the second question would at the same time answer the others.

Before proceeding further it is necessary to define the words "cilium" and "flagellum". A cilium is a fine vibratile thread projecting with many others from the surface of a cell. Cilia lash in an orderly beat in a constant direction. The beat consists of an effective stroke and a recovery stroke. In the effective stroke the cilium is stiff and it drives the water ahead of it; in the recovery stroke the cilium is more flexible and the tip of the cilium follows a lower curve. It is of interest that the difference in flexibility may be retained some time after the cilium has stopped: when moved with a

needle the cilium appears rigid if moved in one direction and limp if moved in the opposite direction [4]. The force exerted on the water by the cilium is in a plane perpendicular to its length.

A flagellum is a fine vibratile thread projecting from a cell; there are normally only one or two flagella on a cell. The flagellar beat consists of the formation of waves that propagate along the length of the flagellum—either from the base to the tip or in the reverse direction. The water is pushed along the length of the flagellum. In some cases it has been noted that a defective flagellum is capable of forming stationary waves only, these flagella will not propagate the water.

There are many similarities between the flagellar beat and the ciliary beat. In both cases the beat is in one plane, although successive beats of a flagellum may be in planes that rotate along the length axis of the flagellum [9]. In the cilium as well as in most flagella the beat can be described as a bending movement starting at the base and transmitted to the tip. In the flagellum the propagated waves follow each other closely and are fairly symmetrical; the flagellum might therefore at each instant take the shape of a sine-wave (one wavelength long in the case of the sea urchin sperm tail [9]). One implication of this, among others, is that the inner side of the cilium may be shorter than the outer one at the end of the effective stroke; the two sides of the flagellum can retain their resting length throughout the movements. It should be mentioned that cilia and flagella are active units generating their own mechanical energy, they are not passively moved by units within the cell body [8].

The comparatively simple movements performed by the cilia and the flagella would not seem to require a very complicated type of machinery. Therefore it has been astonishing to find that the machinery of cilia and flagella is quite complicated indeed; the reasons for this are by no means clear.

Figure 5 (p. 562) is a cross-section through three sea urchin sperm tails. This figure gives us a view into the motor units of the flagellum. The appearance of the nine peripheral double filaments and the two central ones has been described in an earlier communication [1]. The peripheral filaments have projections called "arms" and "spokes", and these projections belong to one of the two subunits of the filaments. There is a complex bridge formed between two of the peripheral filaments. The filament opposite this pair is called the unpaired filament (filament 1).

The filaments of the sea urchin sperm tail are fixed proximally at a disc in the basal body, and end freely distally as separated filaments at the tip of the tail.

Figures 1 and 2 are from another type of flagellum—the tail of the squid spermatozoon. When the spermatozoon is actively swimming this flagellum shows movements which are similar to those of the sea urchin

sperm tail, but at the end of the life span of the spermatozoon the flagellum may perform asymmetrical twitches of a non-propagating type. A twitch consists of a slower bending phase and a more rapid straightening phase which is directed towards the asymmetrical midpiece [3]. Figure 1 shows two squid spermatozoa in which the section includes the two midpieces

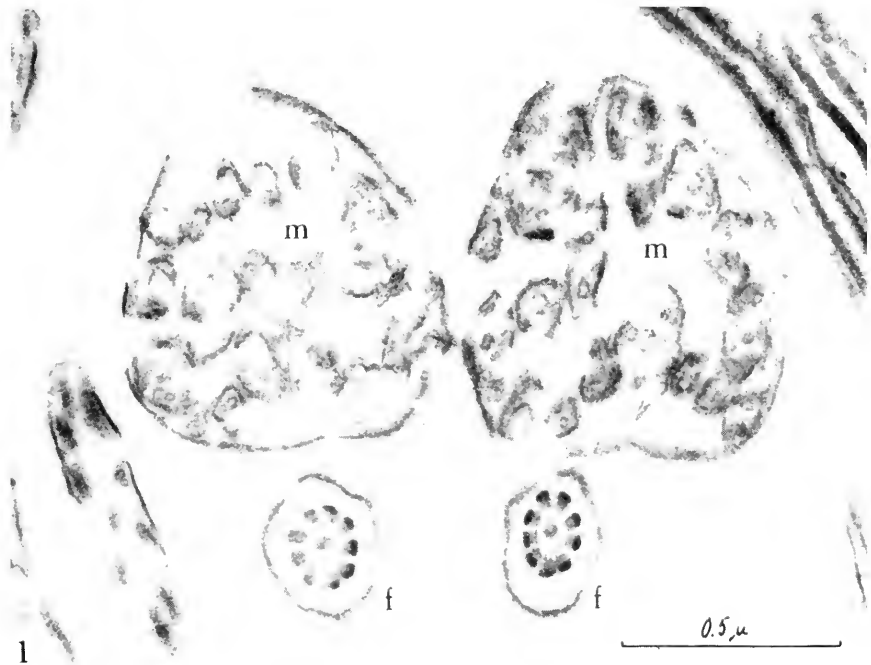


FIG. 1. Cross-section of two squid spermatozoa (*Loligo pealii*). The respective midpieces (*m*) and tail flagella (*f*) are included in the section but not the heads. There is a marked separation between the midpieces and the tail flagella owing to an asymmetrical position of the midpiece in relation to the centre axis (i.e. the tail). It is of interest that the arrangement of the 9 + 2 tail filaments is fairly constant in relation to the midpiece: a line through the two central filaments is at right angles to the line connecting midpiece and tail; the outer nine filaments are unevenly spaced in the ring around the central ones as the two filaments located away from the midpiece are closer together than any other two peripheral filaments. Magnification 57 500 ×.

and below them the respective flagella. It can be noted that the arrangement of the flagellar filaments is fairly constant and that the unpaired filament 1 is closest to the midpiece. It may be inferred from pictures like this that the arrangement of the filaments is fixed in relation to the midpiece and that thus, the direction of the flagellar twitch is fixed in relation to the arrangement of the filaments.

The interest in Fig. 2 lies in the two tail cross-sections that are properly

cross-cut. In these flagella the peripheral filaments can be seen to contain subunits of different electron density. The subunit provided with "arms" and "spokes" appears dark, the other one appears light. Similar findings have been described from observations on some mammalian (Fawcett, personal communication) and an avian [14] spermatozoon.

The next type of flagellum to be described is that of the sponge (*Microciona* sp.) collar cell (choanocyte). According to Kilian [12] the choanocyte



FIG. 2. Cross-section through four squid sperm tails (*f*). The left tail is sectioned close to the centriole and is partly surrounded by the nucleus (*n*). In this flagellum and in the one to the right a proper orientation of the section has allowed a detailed study of the flagellar filaments. The nine outer filaments are connected to nine electron-dense accessory filaments that form a circle outside the proper flagellar filaments. The nine flagellar filaments are themselves double, in that they can be said to consist of two subunits, one having a light and the other a dark appearance. Magnification $81\ 250\times$.

flagellum works with a regular flagellar beat when the collar is expanded but with a beat similar to that of cilia when the collar is retracted. Figure 3 shows at low magnification a section through a chamber lined with collar cells. The marked area in this picture is further enlarged and is shown in Fig. 4. Two notable features characterize this flagellum with regard to its fine structure: (1) There is a marked difference in size between the two subunits of each of the nine peripheral filaments. The larger subunit is that which is provided with arms; a reverse proportion has been found in some multiflagellated protozoa [7]. (2) There are thin indistinct hairs lining two sides of the flagellum (marked *h*). These hairs are roughly in a line parallel to the two central filaments. The appearance is similar to that

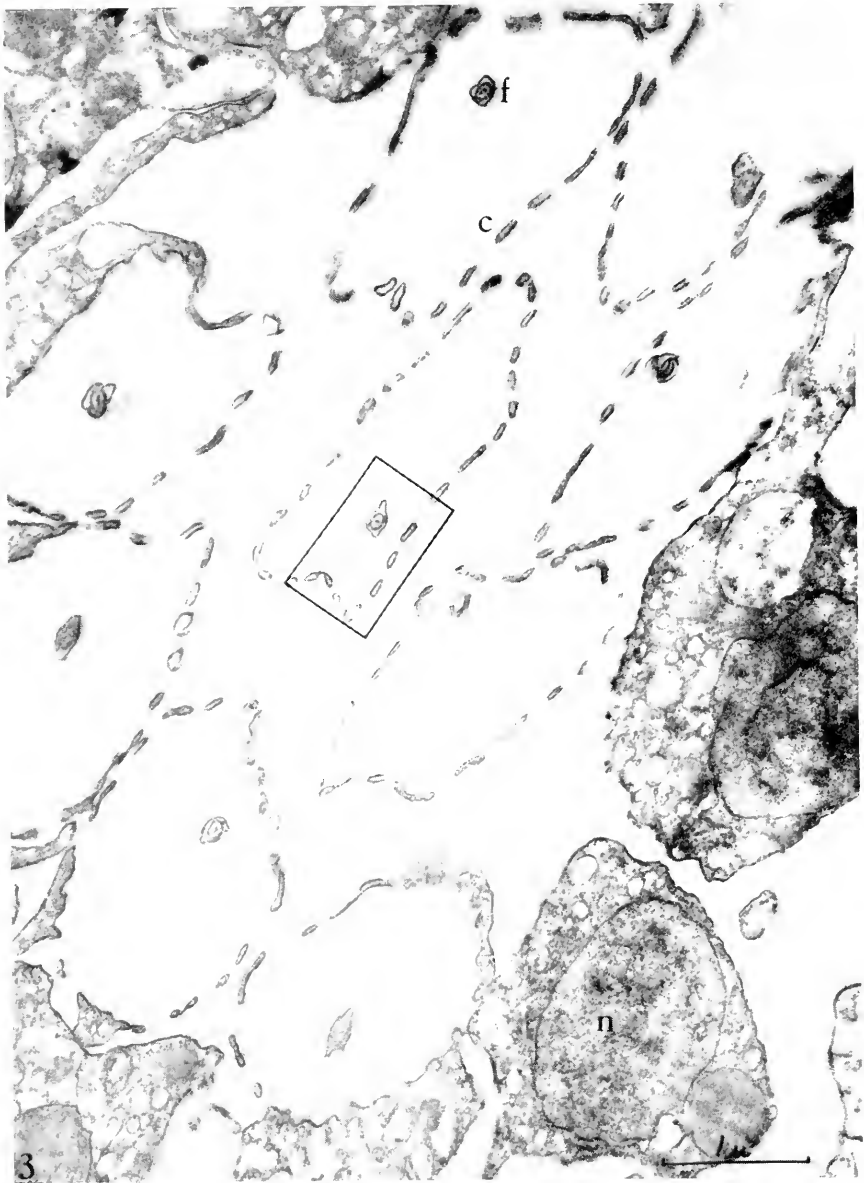


FIG. 3. Section through a flagellated chamber of the marine sponge, *Microciona* sp. Nine collar cells (choanocytes) have been cross-sectioned. In each of them the flagellum (*f*) and the collar (*c*) can be seen. The collar is composed of about 30 separate microvilli. An enlargement of the marked area appears in Fig. 4. Magnification 20 000 \times .



FIG. 4. An enlargement of a portion of Fig. 3. The flagellum has hair-like appendages (*h*) and can thus be regarded as a so-called flimmerflagellum. The hairs have lateral positions (i.e. a line can be drawn through the two inner filaments and through the hairs). Magnification 105 000 ×.

FIG. 5. Transverse section through three sea urchin sperm tails (*Psammechinus miliaris*). The detailed morphology of this flagellum has been described in an earlier communication [1]. Magnification 80 500 ×.

FIG. 6. Longitudinal section through the basal parts of two laterofrontal cilia from mussel gill (*Mytilus edulis*). The central filaments stop at a transverse basal plate. The peripheral filaments continue (arrow) through this plate and enter the basal body of the cilium. The cell borders are visible at *b*. Magnification 56 000 ×.

of so-called "flimmerflagella" which have been described in plants by Manton [13].

We will now turn to cilia. One animal seems to have been used more often than any other in studies of ciliary movement, namely the mussel, *Mytilus edulis*. The gills from this animal seemed suitable for examination in the electron microscope although there are several different types of cilia in a mussel gill. Figures 6, 7 and 8 represent some of the types present. In Fig. 8 the section passes near the tips of the gill cilia, and it can be seen that the peripheral filaments are single. As in sea urchin sperm tails there is no evidence here that the individual filaments join close to the tip. In Fig. 7 which represents another type of gill cilia there is on the other hand a top plate in which the eleven filaments fuse. A similar distal fusion of the peripheral filaments have been described by Rhodin and Dalhamn [15] in cilia from rat trachea. Figure 6 represents a longitudinal section through the basal parts of two "laterofrontal cilia". The peripheral fibres can be followed from their more distal parts down through the "basal plate" (arrow). They terminate at some distance below this plate (cf. ref. [6]).

The last two figures (Figs. 9 and 10) are cross-sections of a unique type of cilia which constitute the ctenophore swimming-plate (*Muevniopsis leidyi*). The cilia are very long and a great number of them are fused together. Their fine morphology is equally unique, and the filament pattern can be described as $9+3$ in contrast to the usual $9+2$ (Fig. 10) [2]. There are ridges in the cilia which join two of the peripheral filaments to the cell membrane—and in many instances seem to connect filaments in neighbouring cilia through a similar substance between the ciliary membranes (arrows in Fig. 9). These ridges presumably represent the morphological equivalent of the phenomenon of ciliary fusion.

We have now some information on the structure of cilia and flagella, and we have some information on their function. We have two types of information, but these two types do not seem to fit together well. There is no simple answer to the question of ciliary movement. At the present time one is tempted to propose temporary working hypothesis by finding analogies in other systems that are better understood. The most obvious analogy is the contraction of a muscle. Perhaps the filament-sliding hypothesis of Huxley and Hanson [11] may serve as a model. Biochemically the work of muscles and the work of cilia and flagella appear similar although not identical [8, 10].

The bending of a cilium or a flagellum must consist of a contractile element as well as of an element capable of resisting compression, an elastic backbone [9]. It seems likely that the nine peripheral filaments are contractile units. The "arms" have a certain resemblance to projections on the myofilaments [1]. As the nine filaments are continuous throughout

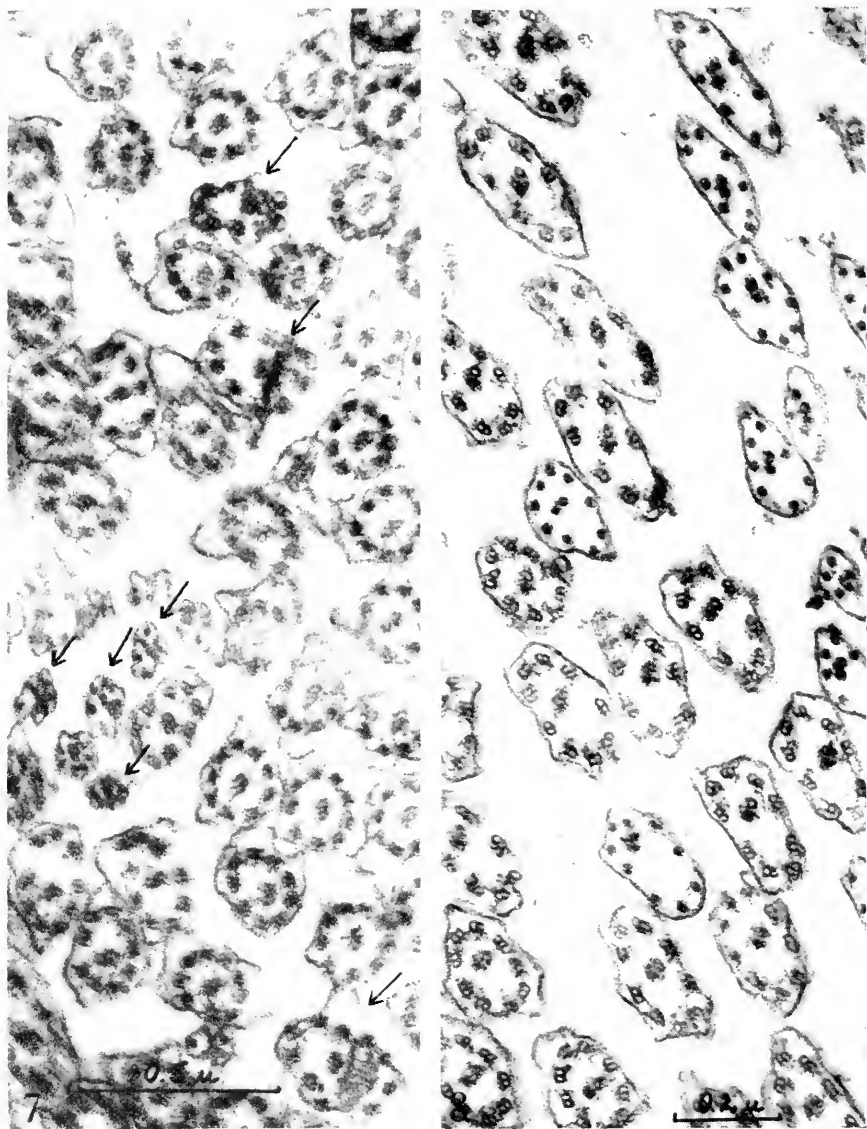
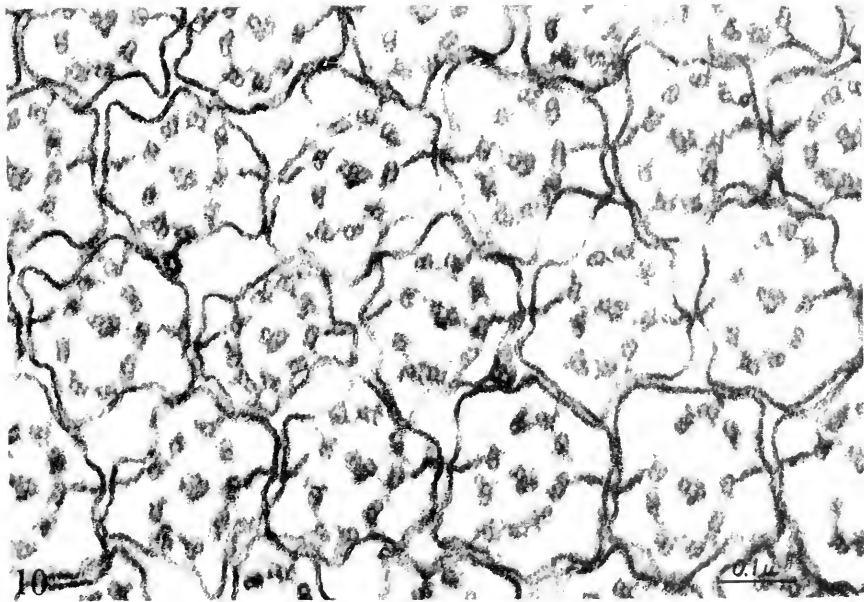
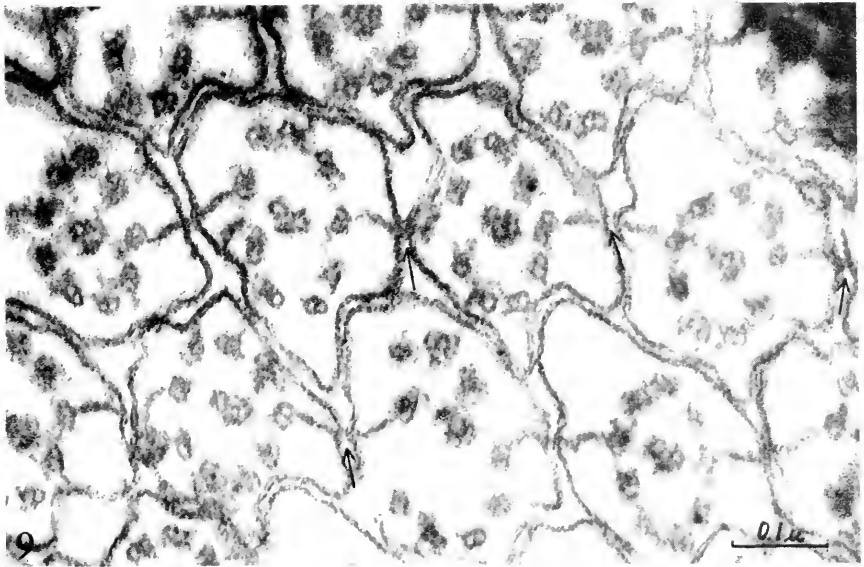


FIG. 7. Transverse section through cilia from mussel gill (*Mytilus edulis*). The arrows point to cilia where part of the "top plate" has been included. The 9 + 2 filaments seem to fuse in the top plate in the distal part of the cilium. Magnification 54 000 ×.

FIG. 8. Another transverse section through cilia from mussel gill (*Mytilus edulis*). This type of cilium differs from that in the preceding figure by having no top plate. The nine peripheral filaments are single in their distal tips and do not join with each other. Note here also the asymmetrical position of the two inner filaments in most of the cilia. Magnification 68 000 ×.



FIGS. 9 AND 10. Cross-sections through a small portion of a swimming-plate from the ctenophore, *Mnemiopsis leidyi*. The filament arrangement is $9+3$ rather than $9+2$, as there is a compact centre filament close to the two tubular ones. Two of the nine filaments in the outer ring are connected to the ciliary membrane by a ridge, visible as a line from these lateral filaments to the ciliary surface. The arrows in Fig. 9 point to places between the ciliary membranes where there can be seen a bridging substance joining the cilia. These bridges are close to the attachments of the ridges. Magnifications $125\ 000\times$ and $105\ 000\times$, respectively.

the length of the cilium a contraction by sliding would seem possible only if one of their ends is fixed and the other end free to move. The findings presented here show that the free end might be either at the tip or at the base of the cilium, or the flagellum. There is no correlation with the direction of the propagated wave. As the nine peripheral filaments follow straight paths [5, 15, 16] the filaments will have an unequal degree of contraction (or sliding) in the uniplanar beat. In this connection it is of particular interest to consider the possibility of lateral fusion of the swimming-plate cilia by means of ridges from the "lateral" filaments. These filaments would thereby be unable to contract or move during the ciliary beat.

The two central filaments are likely to be the candidates for the function of an elastic backbone. Their position and morphology indicate that they have this function and that they may determine the direction of the beat. It has been shown that the inner filaments are in a line perpendicular to the direction of the beat in mussel gill cilia [11], in ctenophore cilia [2], and, as stated above, in the flagellum of the squid sperm. This is probably also true of the choanocyte flimmerflagellum; only when the flagellar beat is perpendicular to the line through the inner filaments will the hairs be helpful in increasing the effective area of the flimmerflagellum.

We are beginning to understand a little of the arrangement of the filaments in cilia and flagella, but when it comes to an explanation of the basic mechanism of ciliary (and flagellar) movement we are still left without an answer.

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Discussion

GOLDACRE: Is there any direct evidence that these (9+2) filaments are the motile element? Have they ever been fixed and viewed in the contracted state?

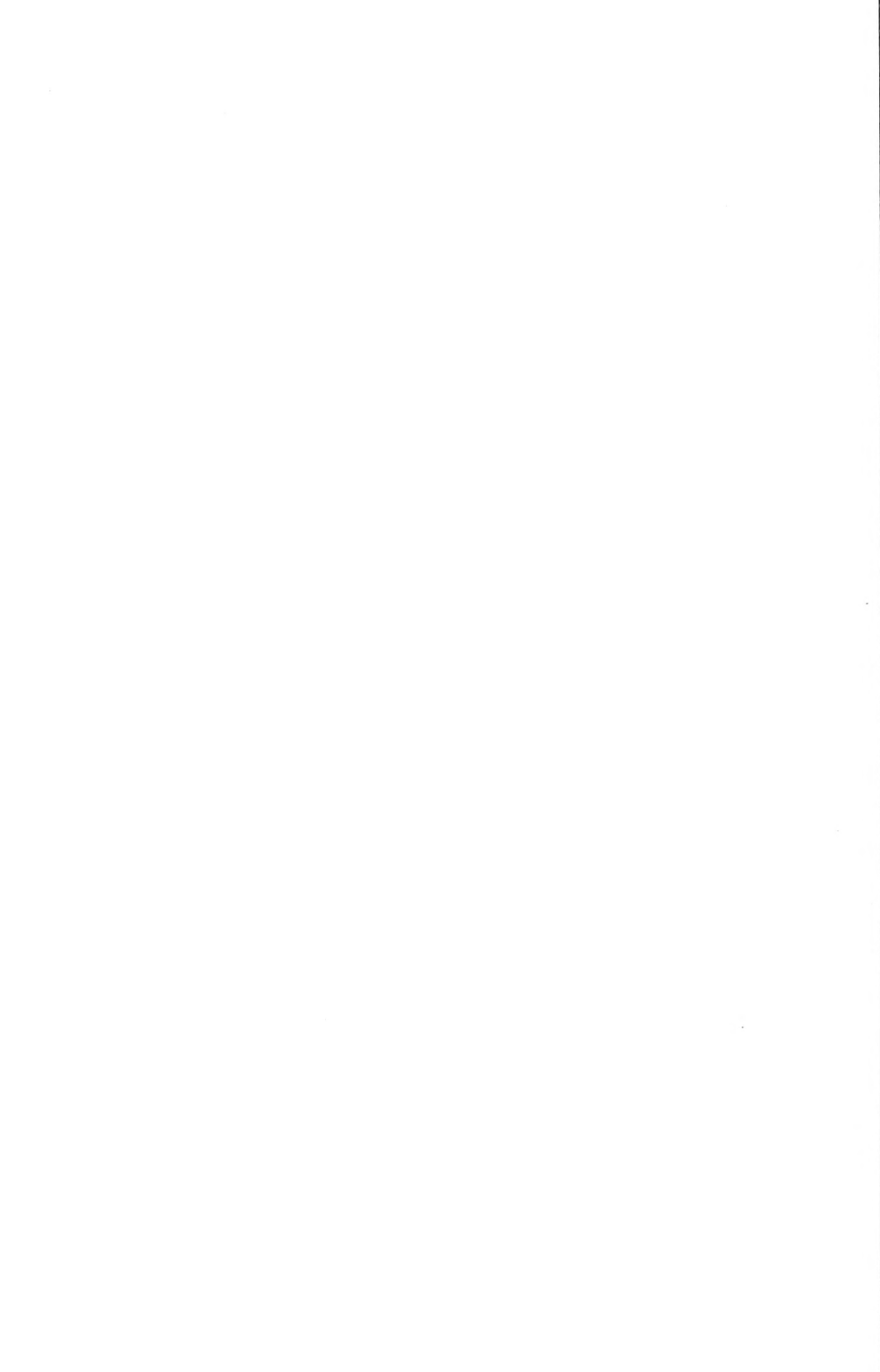
AFZELIUS: No. It is obviously important to investigate a ciliated epithelium in which the metachronal waves of the cilia have been preserved. The differences that would be found with regard to the dimensions or the mutual positions of the filament would probably tell us much of the mechanism of the cilia. This project is, however, not as simple as one would expect. I have not been able to fix the metachronal waves. At present it is not known what is the contracted state of a cilium, or even whether the terms contraction and relaxation can be applied to certain phases in ciliary and flagellar movements.

SHELDON: In the light of recent experiments from Portugal and looking at your picture do you think the cilia are oval or round?

AFZELIUS: You are talking about the paper by Serra in the last issue of *Experimental Cell Research* [20, 395 (1960)]. I think the ciliary cross-section is round. The author might however be correct when he emphasizes that there are other factors than the mechanical work of the cilia that determine the morphology of the cilium.

RUNNSTRÖM: What other factors will there be?

AFZELIUS: According to Serra the mode of duplication of the basal body determines some features of the ciliary structure.



SPECIFIC MEMBRANE TRANSPORT AND ITS
ADAPTATION



Chairman's Introduction

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In analyzing metabolic pathways it has long been profitable to approach the cell as though it were merely a bag of enzymes. Recent years, however, have seen a broad and rapid increase of interest in the properties of cell membranes. Two of the reasons have already been prominent in this Symposium: the intracellular detail revealed by the electron microscope, and the dependence of mitochondrial function on an organized relation of enzymes to membranes. But probably the most dramatic contribution has come from bacteria: not only do these cells also possess a variety of specific transport systems, but these systems have been found to respond, like the intracellular enzymes, to control by induction, repression, and mutation. This development provides compelling further evidence for the reality and the importance of specific transport systems; even more, it offers hope of a new approach to their understanding.

The evidence for specific transport systems is rather indirect, compared with that available for most biochemical entities. Hence inferences involving them have often met with scepticism. Indeed, the various kinds of evidence available for these systems are each usually capable of alternative interpretations, and it is only the convergence of a number of lines of evidence that has now led to quite general acceptance. Since these various kinds of evidence may not all be familiar to biochemists, and since the unfortunate absence of two scheduled speakers has given us extra time, I shall try to summarize the evidence briefly.

But first I would like to devote a few minutes to historical and to comparative considerations. For most of the work on permeability in bacteria has developed autochthonously, rather than as a product of laboratories concerned primarily with permeability; hence it has tended somewhat to neglect the unity of biology at a molecular level, which so dominates our thinking throughout biochemistry today.*

* In this connection, however, it should be noted that electron microscopy reveals for the cytoplasmic membrane of bacteria only a single dark and a single light layer, whereas the membranes of all other organisms studied have shown, following similar fixation, a light layer between two dark layers. Furthermore,

Early studies on cell membranes naturally focused on their resemblance to the simple physicochemical models provided by artificial semipermeable membranes, in which penetration took place by diffusion through pores. And, indeed, the kinetics of the penetration of certain substances into cells, including dissolved gases and some very small organic molecules, could be accounted for by this mechanism. Many large molecules, however, exhibited anomalously high values; and studies of certain homologous series showed a parallelism between rate of penetration and lipid solubility (which increased with size). Hence penetration by solution in lipids, in which biological membranes are known to be rich, was recognized as another significant mechanism. Both these mechanisms were compatible with the view of a biological membrane as a relatively homogeneous undifferentiated structure—perhaps a somewhat porous double layer of protein and lipid.

Nevertheless, the behaviour of most of the metabolically important substances that have to penetrate into cells, including sugars, amino acids, and inorganic electrolytes, did not fit either of these mechanisms; and a major development in the nineteen-thirties was the recognition (at least among the band of specialists in this field) that with most substances penetration into cells involves specific transport systems. In contrast to the diffusion mechanisms previously described, the rate of transport by these specific systems does not increase indefinitely as a function of permeant concentration but instead exhibits saturability, implying a mass-law interaction between permeant and transport system. This conclusion implies functionally differentiated regions of the membrane. It is this aspect of the cell membrane, rather than its generalized or average properties, that now seems to deserve most attention, much as the study of specific enzymes and intracellular organelles has displaced the study of "protoplasm".

Two groups of specific transport systems have been recognized. The first, of which the sodium pump is an example, can carry out active transport—that is, it can move its permeant to a region of higher thermodynamic potential. The second group, such as those responsible for entry of sugars into erythrocytes, cannot transport uphill: with a non-metabolized substance these systems can only accelerate the approach to equilibrium (i.e. to the same chemical potential on both sides of the membrane), and with a metabolizable substance the rate of utilization is accelerated.

This second kind of specific transport has been called *facilitated diffusion* by Danielli. Though this term is widely used it does not seem

bacteria are unique in lacking steroids. It is therefore quite possible that future analysis will reveal significant differences as well as broad similarities in the structure and in the function of transport systems in bacterial compared with other biological membranes.

ideal; for (a) the process does not obey the kinetics of diffusion; (b) while any transport must involve motion of something from here to there, we are still so ignorant of the mechanism that any emphasis on its resemblance to diffusion may be prejudicial; and (c) the term suggests a mechanism very different from that of active transport, whereas it is quite conceivable that the same "ferryboat" may be capable of either working at active transport or coasting along uncoupled from energy expenditure, depending on the concentration of permeant. It might therefore be worth considering a classification of *specific transport* into *active transport* and *passive transport*; these terms seem as neutral as possible with respect to mechanism, and they are clearly distinguished from non-specific permeability due to direct diffusion (either through lipid or through an aqueous pore) rather than to transport by some sort of carrier.

What is the evidence for the existence of specific transport systems—and of the corollary impermeability of a membrane to substances for which such a system is lacking?

I. Crypticity

With the discovery of more and more enzymes many cases have been recognized, in all kinds of biological material, in which cells showed little or no enzyme activity when intact but became active after mechanical disruption or after chemical damage to the membrane (e.g. by toluene). While this crypticity clearly suggested a permeability barrier, the evidence was not rigorous; for the phenomenon could also conceivably be due to the presence of the enzyme in the cell in a masked or inactive form.

Late developments with bacteria produced one case in which the latter alternative could be excluded. The well-known inability of many bacteria to utilize citrate (or certain related members of the tricarboxylic acid cycle) might be due to a permeability barrier or to absence of the required enzymes. However, citrate was shown to be an obligatory intermediate in the biosynthesis of glutamate from glucose in *Aerobacter aerogenes* [1]. Since cells could be shown to be unable to utilize *exogenous* citrate under conditions where they must be rapidly metabolizing *endogenous* citrate, a permeability barrier to citrate could be inferred [2].*

* Reliance on studies with intact cells, combined with scepticism concerning the possibility of a permeability barrier to citrate, was responsible for prolonged doubt among many investigators concerning the existence of the tricarboxylic acid cycle in microbes. In retrospect, indeed, it is rather ironical to find Professor Sir Hans Krebs himself among this group [3]. Similar barriers did not interfere with the recognition of the cycle in mammalian cells. Reconsidering this difference, one is led to wonder whether bacteria, growing often in highly dilute environments, might not need to retain tenaciously their intracellular pools of essential intermediates (such as those of the tricarboxylic acid cycle), whereas the environment of the mammalian cell might make this requirement unnecessary. In a related

2. Active transport

This phenomenon *per se* implies specific transport; it also implies that the remainder of the membrane, aside from the "pumps", must be relatively impermeable to the substance. But the analytical determination of an elevated concentration of a permeant in a cell does not necessarily prove active transport; it could equally well reflect binding to cell constituents (which has often been invoked). Such binding, however, cannot explain the high osmotic pressure of bacteria and plant cells relative to their environment. Neither can it explain the striking difference in concentrations of specific electrolytes found in intracellular and extracellular fluids of higher animals, nor the evident ability of all kinds of secretory and excretory organs to perform osmotic work. An additional argument put forward by Cohen and Monod [8] is based on the properties of bacteria that can take up but not metabolize lactose: such cells can reach intracellular levels of the compound as high as 20% of the dry weight of the cell. This result could be accomplished by a small number of catalytic pumps but would require an implausibly large number of stoichiometric "hooks"—all the more implausible since the capacity for active concentration, as shown below, could be entirely eliminated (or made to appear) by growth for a few generations under conditions of repression (or induction). It should be noted, however, that while macromolecular "hooks" are thus excluded, conversion of permeant to a labile low-molecular-weight derivative is not.

It is of interest to note that the capacity of bacteria to concentrate amino acids, discovered by Gale [5], appeared to be restricted to Gram-positive organisms, such as *Staphylococcus*. The later work of Cohen and Rickenberg [6] showed that the same phenomenon can be observed also in Gram-negative organisms such as *Escherichia coli*, but these require greater precautions to avoid washing the permeant out of the cells before analysis.

3. Kinetics

As noted above, the rate of initial penetration of most substances that have been studied, plotted as a function of concentration, yields the mass-law curve that would be expected if the penetration required formation of a carrier-permeant complex, and if the rate of penetration was proportional to the concentration of that complex. This is precisely analogous to the classical "Michaelis" kinetics for enzyme action. Observations of this kind on erythrocytes provided the main basis for the recognition of specific transport systems for substances that were not actively concentrated.

consideration, it has been suggested that the need for retaining intermediates may be responsible for the curious fact that the biosynthetic paths developed in the course of evolution involve almost exclusively ionized compounds [4].

Studies on kinetics involve not only rates of transport but steady-state levels. In the recent work on uptake by bacteria of the non-metabolized β -galactoside TMG (β -methylthio-D-galactoside) it has been shown that the levels reached in active transport, at various concentrations of permeant, appear to depend on a steady state between entrance by a specific pump and exit by diffusion, or at least by a system exhibiting the linear concentration relations of diffusion [7, 8].

4. Competition

Studies with radioactive compounds in animal cells and in bacteria have shown that certain structurally related permeants (e.g. similar amino acids such as isoleucine and valine) interfere with each other's entry, in terms both of rate and of final level reached. This finding is incompatible with diffusion through pores, but consistent with either entry via a common carrier or adsorption to common intracellular sites. With β -galactosides in bacteria, it has been possible by further competition studies to choose between these two mechanisms [7, 8]. The active transport system for β -galactosides exhibits both different Michaelis constants and different rates of transport for various members of this class of compounds. When the cells are in equilibrium with permeant A the addition of B, with higher affinity and slower transport than A, displaces several molecules of A per molecule of B taken up. This finding fits competition for a transport system but would be difficult to reconcile with competition for intracellular binding sites.

This competition has clarified certain obscure cases of analogue inhibition. Analogues of metabolites, such as the sulphonamide drugs, have generally been considered to inhibit growth by competing with the corresponding metabolite at an enzyme site. However, some analogues, notably of amino acids, interfere with exogenously added metabolite but not with the same metabolite endogenously formed. Thus arginine inhibits the growth of mutants that require lysine but not of the parent strain, which synthesizes its own lysine. The prolonged blindness of non-specialists to permeability problems is illustrated by the fact that this lysine-arginine problem perplexed all of us interested in microbial mutants for a decade, until Mathieson and Catcheside [9] suggested the now obvious explanation and supported it with evidence that arginine interfered with lysine uptake.

5. Mutation

A novel contribution of studies on bacteria was the finding that the formation of a specific transport system, like that of an enzyme, was under the control of a corresponding gene. Suggestions in this direction arose from explorations of biosynthetic pathways with auxotrophic mutants,

which provided many examples of apparent intermediates that could not serve as a growth factor, presumably because of a permeability barrier. In at least two cases, involving mutants blocked before 5-dehydroquinone [10] and citrate [2], it was possible to select secondary mutants that had gained the ability to grow on the intermediate. Since the required enzymes were already present in the cell before this second mutation, it seemed evident that a one-step mutation had altered the permeability properties of the cell.

A much more extensive exploration of mutational effects on a transport system has been provided by Cohen and Monod for the β -galactoside system [8]. It has been demonstrated that the gene controlling the formation of the transport system and that controlling formation of the enzyme β -galactosidase are distinct though closely linked on the chromosome: a mutation can prevent or restore the formation of either without affecting the other. Of particular interest, for present purposes, are two properties of a cryptic mutant, i.e. one which retains β -galactosidase but is transport-negative for β -galactosides:

(1) Compared with a transport-positive, the transport-negative strain metabolized β -galactosides very much more slowly; and the relation of rate to substrate concentration implied that the rate-limiting step in this strain is diffusion rather than the action of a system characterized by a Michaelis constant. We thus see that in the absence of a specific transport system the same permeant can penetrate slowly, presumably via a more primitive mechanism.

(2) In addition, the transport-negative strain had lost not only the capacity to metabolize lactose rapidly but also the capacity to concentrate a non-metabolized β -galactoside (TMG). This finding is important in linking studies on active concentration and those on rate of substrate utilization to the same functional unit. For the loss of apparently active concentration could conceivably also be due to loss of ability to convert the permeant into a labile intracellular derivative, and loss of rapid utilization could conceivably be due to formation of the intracellular enzyme in a masked form; but only loss of a specific transport system could singly account for both effects of the mutation.

Incidentally, metabolic inhibitors such as azide eliminated the active concentration but not the rapid utilization, suggesting that the same specific transport system, which requires an energy supply for function in active transport, may in the absence of an energy supply still function in passive transport [8].

6. Induction and repression

Another novel contribution from bacteria was the finding that in an appropriate cell the presence of certain transport systems, like that of certain enzymes, requires induction by growth in the presence of the

substrate (or an analogue), and can be repressed by the presence of a preferred foodstuff such as glucose. Induction of citrate transport in *Pseudomonas* [11, 12], and induction and repression of citrate transport in *Aerobacter* [13] and β -galactoside transport in *Escherichia coli* [7, 14], were discovered independently in four laboratories. It is of interest to note, in all these early communications, reluctance to trust the conclusion, however logically derived from the evidence, that the properties of a cell membrane could be substantially modified by the nature of the growth medium. Apparently such a flighty disposition, responsive to suggestions from the environment, was easier to ascribe to invisible molecules in the cytoplasm than to a solid, microscopically visible structure like a membrane!

These, then, are the major kinds of evidence available for the existence of specific transport systems. The control of these systems that is possible in microbes has, of course, opened up new avenues of approach to their nature. One of the most significant findings has been that the formation of the inducible transport systems for citrate [13] and β -galactosides [7, 8], like the formation of inducible enzymes, requires conditions that permit protein synthesis. We thus have strong evidence for a proposal offered by earlier permeability workers on more speculative grounds (cf. [15, 16]): that the specificity of transport systems must depend on the presence of proteins with a specificity similar to that already familiar in enzymes. It has also been possible, by varying the number of "pumps" per cell (through partial induction), to analyze more deeply the kinetics of entry and exit in a system carrying out active transport.

Monod has proposed the term "permease" for specific transport systems, whether active or passive [7, 8]. This term has the advantage of focusing attention on a most important property of these systems: the presence of elements which resemble enzymes in their specificity, in their mass-law relation to substrate, and in the genetic and environmental factors influencing their formation. The term, however, has serious disadvantages. First, being based on the historical discontinuity between studies on bacteria and those on other cells, it has been construed as implying entities quite different from the "carriers" proposed by Danielli, Le Fevre, Widdas, Wilbrandt, and Ussing (cf. [17]) to account for specific transport in animal cells. In fact, however, the *functional* properties of the two systems are essentially indistinguishable; the novel feature of the bacterial systems has been the possibility of controlling their *formation*. Until proved otherwise, it would seem wiser to assume that the specific transport systems of all cells are fundamentally similar; a unified terminology would thus be desirable. And while "permease" has been widely used with reference to bacteria, it does not seem to have been extended to other cells.

A second, more serious objection to the term "permease" is its implication that the transport system *is* an enzyme. Here a good deal of history, from zymase to methionine synthase, has sensitized biochemists to the distinction between an enzyme and a more complex system. Finally, while one can argue whether or not the term "enzyme" should be restricted to catalysts that change a covalent bond in a substrate, there is general agreement that the term is not usefully applicable to such proteins as haemoglobin, which only form a loose, reversible association with their substrate. And we must at present not restrict our thoughts on models for specific transport to those in which the permeant is enzymically converted into another compound at one side of the membrane and restored again at the other side; we must also be willing to entertain models in which the permeant is only loosely associated with a carrier which shuttles or rotates back and forth. The latter models, indeed, would better fit the possibility that organic compounds and inorganic ions are transported by similar systems. For these several reasons the writer prefers, instead of "permease", the less committal term "transport system".

In closing, I would like to list some of the problems concerned with permeability that now press for analysis at a molecular level. What is the structure of transport systems, and how are the specific carrier proteins related to the lipid in these differentiated portions of the membrane? What is the mechanism of the energetic coupling required for active transport? Does it involve change in the structure of the permeant (more than simply reversible adsorption), or change in the structure (and hence affinity) of the carrier, or still another process that will have to be described in as yet unknown terms? Does a system capable of active transport become uncoupled from energy expenditure when transporting downhill rather than uphill? Is the same polypeptide chain, differently attached, responsible for the specificity of a transport system and that of an enzyme acting on the same compound? What accounts for the fact that exchange of external and internal permeant is faster than net transport: does a loaded ferryboat shuttle faster than an empty one? How do compounds normally impermeable from the outside become readily excreted by mutants blocked after them? How much does the non-specific "leakiness" of membranes vary with physiological state, and what is its relation to cellular function and to viability? Is the site of induction of a transport system within the cell? Or is it at the membrane, as suggested by the fact that citrate can induce a transport system for itself in a cell which is relatively impermeable to it, and which is meanwhile rapidly synthesizing and converting citrate endogenously.

In a sense, research on transport systems, despite its spurt during the past decade, has been frustrating. Direct chemical attack on simplified systems, extracted from the cell, has been extending the solid march of

biochemistry from low molecular weight intermediates to macromolecule biosynthesis and even to the structure, function, and synthesis of what might be thought the deepest secret of biology—the gene. But with membranes function is even more intimately related to structure. When one tries the usual biochemical approach of first chopping the material up, normal function, which requires separation of two aqueous phases by the membrane, disappears. Here, more than in most of cell physiology, Goethe's awed attitude toward Nature still applies:

Und was sie deinem Geist nicht offenbaren mag,
Das zwingst du ihr nicht ab mit Hebeln und mit Schrauben.

Nevertheless, it is clear that I have exaggerated for rhetorical purposes. Certain fruitful approaches to various aspects of the problem will be described by this morning's participants, and Dr. Holter has already introduced the phenomenon of pinocytosis. Among other recent encouraging biochemical developments, not represented here, it has been observed that stimulation of the activity of secretory glands is associated with increased phospholipid turnover [18]; and from erythrocyte membranes there has been separated an ATPase that is activated by K^+ plus Na^+ [19]. Finally, since the formation of specific transport systems in bacteria is readily subject to experimental control, there are as yet unexploited possibilities for comparing directly the properties of two membranes which should differ only with respect to a single system. In complex problems of biology, as genetics has particularly shown, we can learn a great deal from studying discrete differences in a single component long before we have learned how to isolate it.

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Approaches to the Analysis of Specific Membrane Transport

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Now that we have reached the last session of this Symposium, I notice that the number of delegates has somewhat decreased, and this prompts me to begin with some remarks about a fundamental thermodynamic concept known as "escaping tendency". The escaping tendency of a particle such as a molecule or an electron or a chemical group describes the tendency of the particle to escape from one place and pass to another by the thermodynamic process of diffusion. As a matter of fact, we are not accustomed to thinking of people as having an escaping tendency in this sense (at least, not a measurable one), for escape in the present context can only occur by thermal movement, and thus the escaping tendency can only be measured when the free energy necessary to move the particle is not very much greater than the thermal vibration energy for each degree of freedom. Nevertheless, every kind of transport process not involving the absorption of radiant energy is primarily caused by diffusion. It may be the diffusion of the molecules of hot gas that propel the piston or turbine blade or air stream of the internal combustion or other heat engines that are at this moment transporting some of our colleagues away from this lecture theatre; it may be the diffusion of the filaments of actin and myosin over one another in our hearts or skeletal muscles; or it may be the diffusion of group donors to a glycosidase or synthetase located in a membrane and the vectorial extrusion of a polysaccharide or other polymer chain from it, as discussed in the session on polysaccharides yesterday afternoon. Except in the case of photosynthesis, wherever there is transport in biological systems it is the result of a spontaneous escape of particles from a higher to a lower free energy state by thermally activated diffusion in space. Of course, this fundamental fact will be very well known to many of those present here, but I feel the necessity to mention it at the outset—to make clearer what I am going to say later—because the concept of what has come to be called "active transport" in biology has sometimes been associated with the idea that the substrate specific transport systems of living organisms can possess a special property that will actually cause

molecules or other particles to pass against the natural direction of the diffusion or escaping tendency, or act, as Cohen and Monod [1] have suggested, as Maxwell demons. This, we can say at least with the certainty of the physicist, is not possible.

Membrane structure and transport function in bacteria

The relative simplicity of the structure of bacteria makes them especially suitable for the study of transport processes at the molecular level of dimensions [2, 3, 4]. In this paper I shall concentrate attention upon bacterial membranes, but will attempt to develop a simple conception of the relationship between transport function and physicochemical structure that may be of general validity in biology.

Broadly speaking there are four main experimental approaches to the analysis of membrane transport which can be summarized under the following headings:

1. Osmotic barrier function of the plasma membrane: General impermeability function; studied by net permeation measurements.
2. Osmotic link function of the plasma membrane: Specific transport function; studied by observations on the specificity and kinetics of the transport process, interpreted in terms of the catalysis of molecular complex-, molecule-, ion-, electron-, and group-translocation.
3. Structure of the plasma membrane: Chemical and catalytic composition; studied by orthodox chemical and biochemical methods.
4. Correlation of structure and function in "synthetic" or reconstituted membrane systems.

The first three of these approaches have been pursued in parallel in my laboratory. Our studies of the osmotic barrier function, beginning with the introduction of the term osmotic barrier 11 years ago [5], can be roughly summarized by saying that in general bacterial plasma membranes are permeable to small molecules carrying three water molecules or less (e.g. glycerol), but they are impermeable to molecules carrying more than four water molecules (e.g. glutamate, phosphate, succinate, and glucose). There are, of course, factors other than the degree of hydration that influence the rate of permeation of different solutes into bacteria. For example, D-ribose permeates much more rapidly than L-arabinose and other pentoses, probably because in the ribose molecule all the hydroxyl groups are on the same side of the ring so that one side of the molecule is hydrophilic while the other is hydrophobic. There are also differences between the permeability of the plasma membrane of different organisms to a given solute. For example, *Staphylococcus aureus* and *Micrococcus lysodeikticus* are quite permeable to alkali thiocyanates while *Escherichia*

coli is not. In general, however, bacterial plasma membranes behave in the way expected of the type of thin lipid film postulated by Overton [6] in his lipid membrane concept at the beginning of this century [7-13, and see 4].

The question naturally arises—and this has been discussed ever since the lipid membrane concept was introduced—if the membrane is impermeable to the nutrients of the medium, how do they get into the cell during metabolism and growth? The kinetic studies of glutamate and lysine transport in streptococci and staphylococci in which I was implicated in Dr. Gale's laboratory [14, 15, 5], and the detailed kinetic analysis of phosphate translocation in staphylococci which I undertook shortly after [7, 16-18], led us to the conclusion that transport systems of high substrate specificity, exhibiting kinetic features indistinguishable from those of the classical enzyme and carrier systems of biochemistry, must be responsible for allowing the nutrients to enter the metabolic systems of bacteria. This conception of the very close relationship between transport and metabolism in bacteria has been confirmed by the more recent studies in my laboratory on sugar and carboxylic acid transport [see 2, 3, 4, 19, 13], some aspects of which I shall describe later in this paper. The kinetic studies of galactoside and amino acid uptake in *Escherichia coli* carried out by Monod and his collaborators [20-22] also support our view of the intimate relationship between the phenomena of transport and metabolism—although, the interpretation which Monod and his collaborators placed on these studies was fundamentally different from ours [1]. According to the recent work on the kinetics of galactoside uptake into *Escherichia coli* described by Kepes [23], the "galactoside permease" system is identical in principle to the hypothetical system originally suggested for the passage of "glutamate" into streptococci through the enzyme-catalyzed conversion of glutamate to glutamine on the cell surface and diffusion of glutamine through the membrane [5]. This is satisfactory in demonstrating the present consensus of opinion as to the most elementary types of molecular mechanism that could be involved in specific membrane transport; but it also shows how loose and unsatisfactory the use of the word "permease" has become, even amongst different workers at the Pasteur Institute. I feel, therefore, that I must digress for a moment to say that I am inclined to associate myself with the suggestion that Dr. Davis made in his introduction to this session of the Symposium, that the word "permease" might best be abandoned. I advocated in the past that the word "permease" should be strictly used to mean a protein catalyst of facilitated diffusion [2-4], and perhaps this use might still be introduced if such a catalyst should be found to exist.

As Dr. Davis mentioned in his introduction, Kogut and Podoski [24], Barrett *et al.* [25], Green and Davis [26], and Monod and his collaborators [21] discovered that the catalysts of the entry of certain carboxylic acids

and sugars into the metabolic systems of *Escherichia coli* and *Pseudomonas* sp. resemble enzyme systems in being inducible, and that the induction can be blocked by certain inhibitors of protein synthesis. These kinetic observations lend further support to the idea that the transport catalysts may be normal enzyme and catalytic carrier systems. But we must be careful not to imagine, as some microbiologists have done, that kinetic and inhibitor studies of the behaviour of whole cells or protoplasts can reveal the composition of the catalysts immediately involved in the transport processes—for example, whether they are proteins or not. The only unequivocal way of characterizing the catalysts of transport is to isolate and purify them, and to examine their structure and function by direct analytical and kinetic methods.

Let us now turn to the third method of attack on the membrane transport problem—the study of the composition of the plasma membrane. This phase of the work has its origins in the isolation of a small-particle fraction from disintegrated micrococci which Dr. Moyle and I found to be a lipoprotein, just sufficient in amount to have originated by the fragmentation of the plasma membrane, and containing an acid phosphatase which, in intact cells, we knew to be accessible to glycerophosphate from outside [27, 18]. I should, perhaps, say at this point that the problem of isolating the plasma membrane material from bacteria is made comparatively easy by the very small size of the cells and their large ratio of area to volume, a membrane only 10 $m\mu$ thick at the surface of the protoplast representing 5–10% of the dry weight of the cell.

The isolation of the membrane material in a morphologically recognizable state owes much to Dr. Weibull's discovery that the cell wall of certain bacteria could be removed enzymically without breaking the plasma membrane as long as the protoplast was prevented from swelling by the addition of sucrose or other osmotically effective solute to the suspension medium [28]. When the suspension medium was suddenly diluted after removing the cell walls from the protoplasts, the contents of the protoplasts were thrown out, and the membranes, looking like little bursted balloons in light and electron microscopy, could be collected on the centrifuge [29]. In this way, Dr. Weibull was able to show that the cytochrome pigments of *Bacillus megaterium* sedimented in the membrane fraction; but as this organism unfortunately contains very many cytoplasmic particles which also sedimented with the membrane fraction, it was not certain whether the cytochrome pigments belonged to the membrane or to the adhering particles. Dr. Moyle and I therefore undertook a similar type of fractionation on staphylococci, which are comparatively free of cytoplasmic particles [30]. We relied upon a controlled autolytic method to weaken the cell wall before diluting the cell suspension to burst the protoplasts and liberate morphologically recognizable membranes. It

was found that the weight of the membrane fraction obtained in this way corresponded quite closely to the weight of our small particle lipoprotein fraction, and we discovered, as shown in Table I, that both the small particles and the intact membranes (from which the small particles were evidently derived) contained not only cytochrome pigments, but many enzyme activities [18, 31]. Similar observations were made soon after by Storck and Wachsman [32] on the membrane fraction of *Bacillus megaterium*, and subsequent studies which Dr. Weibull and his collaborators

TABLE I
DISTRIBUTION OF ENZYMES AND CATALYTIC CARRIERS IN
Staphylococcus Aureus

Enzyme or catalytic carrier	"Soluble" fraction	Plasma membrane or lipoprotein particle fraction
Cytochromes (extinction at 425 m μ)	< 10	> 90
Succinic dehydrogenase	< 10	> 90
Lactic dehydrogenase	5-20	80-95
Malic enzyme	< 10	> 90
Malic dehydrogenase	< 10	> 90
Formic dehydrogenase	< 10	> 90
α -Glycerophosphate dehydrogenase(s)	30-50	50-70
Glucose-6-phosphate dehydrogenase	97	3
Glucose-6-phosphatase	90	10
Acid phosphatase	< 10	> 90

have carried out on this organism suggest that in this case, too, the enzyme activities do actually belong to the membrane complex and are not carried by adhering particles [33]. Dr. Moyle and I extended our work to *Micrococcus lysodeikticus* with results similar to those obtained with *Staphylococcus aureus* (see [2]). In organisms such as *Escherichia coli* and *Azotobacter vinelandii*, where it is very difficult to separate plasma membrane from cell wall material [13, 34], the evidence is perforce less unequivocal, but it seems probable that the so-called insoluble enzymes in these organisms are part of the plasma membrane complex as in the micrococci and *Bacillus megaterium* [35, and see 4].

THE CONCEPT OF TRANSLOCATION CATALYSIS

The fact that certain hydrolytic and oxido-reductive enzymes and catalytic carriers, including those of the cytochrome system, are an integral part of the plasma membrane complex of certain bacteria represents the experimental foundation for our conception of the plasma

membrane as an active participant in the metabolism of the cell as a whole [2, 4]. To admit, however, that the membrane participates in the intracellular metabolic processes is to pose a new question. What, we may well ask, is the function of the enzymes and catalytic carriers that are located in the membrane complex? Why are these metabolic systems organized in the surface of the protoplast instead of being tucked away safely in the cytoplasm? It occurred to me some time ago that this question might be answered in the following way. During group transfer or substrate transfer, the group transfer enzyme or catalytic carrier molecules of classical biochemistry (in as much as they are anisotropic catalysts) catalyze a microscopic vectorial movement or translocation of substrate or chemical group, directed in space relative to the individual enzyme or catalytic carrier molecules. We normally think of metabolism as a scalar substrate and group transfer process (without direction in space) because we think of it as though the enzyme and carrier molecules were orientated at random, so that there would be no macroscopic vector component of the substrate and group translocation processes. But if, as seems likely, the enzyme and catalytic carrier molecules are specifically orientated in an organized membrane structure, the microscopic translocations of the substrates and chemical groups which represent the normal metabolic transfer processes can show as concerted macroscopic transports of substrates and chemical groups (including ions and electrons) across the membrane [2, 36-38]. Thus, we might not need to stretch our imagination very far beyond the bounds of classical biochemistry to conceive how the metabolic systems of the membrane could function as the catalysts and controllers of membrane transport.

You will see now the reason for my opening remarks about escaping tendency and diffusion. We are accustomed to thinking of the diffusion of molecules and the chemical transformation of molecules in rather different terms, but the processes involved in diffusion and chemical change are, in fact, very similar. The diffusion of a solute particle such as a molecule or molecular complex in a biological system describes the movement of the particle by the thermally activated breaking and making of the *secondary* bonds that tend to prevent the displacement of the particle relative to the neighbouring atoms. The chemical transformation of a molecule or molecular complex describes the movement of one of its constituent chemical groups by the thermally activated breaking and making, not only of secondary bonds, but also the *primary* bond that tends to prevent the detachment of the group from its partner (or donor group) and its transfer to an acceptor group. The enzymes and catalytic carriers of a membrane complex must catalyze the movement of molecular complexes, molecules, ions, electrons or chemical groups in the natural direction of the diffusion or escaping tendency. It is convenient to call the catalysis of this natural

process of diffusion down the electrochemical activity gradient "translocation catalysis". When we describe the transport of a substance as "active", it is because we do not know (or do not wish to specify) in what form the substance actually diffuses across the membrane.

SUBSTRATE AND SUBSTRATUM SPECIFICITIES OF ENZYMES AND CATALYTIC CARRIERS

The view that I have been developing of substrate and group translocation as part of the metabolic process in the spatially organized enzyme and catalytic carrier systems of the membrane complex, places the problem of membrane transport in a new light, for it suggests that to picture the process of transport at the molecular level of dimensions we need to recognize, not only the substrate specificities of the enzymes and catalytic carriers as normally understood, but also the locational or substratum specificities that are responsible for bonding these components into the organized structure of the membrane complex [36]. The locational bonds may be said to represent the articulations between the bones of the cytoskeleton [39].

The conception of the bivalent specificity (the substrate specificity on the one hand and the substratum specificity on the other) of the translocation catalysts stems directly from considerations of the transport process *per se*. It is, perhaps, significant that when one considers the transport process from a different angle, that is, in relation to growth and adaptation (as we are asked to do in this session of the Symposium), the same type of conception as I have just outlined seems to be required. For, since it is inconceivable that the catalysts of translocation could all be synthesized at the sites of their activity in the membrane, the very catalytic components of a membrane system that are to cause and control the translocation of a specific substrate must, themselves, possess the specificities that will cause them to be transported to and incorporated in the organized membrane structure during growth and adaptation. As I pointed out some years ago [2, 4], one must take much more care than has been customary in interpreting the results of studies of mutants that lack particular transport capacities, for loss of a transport capacity could as easily be due to a change of locational specificity between a transport-catalyzing enzyme or carrier and its locator region (substratum) in the membrane as to the loss of the catalytic function of the free enzyme or catalytic carrier molecule itself.

I propose now to summarize the results of two series of experiments bearing on the problem of enzyme location and enzyme-substratum specificity that we have recently done in Edinburgh. It has been customary to assume that intracellular enzymes which appear in solution in the

medium, when one breaks the cell wall and plasma membrane of bacteria by methods that do not cause appreciable autolysis, are located within the protoplast of the intact cell. Further, it has generally been assumed that

TABLE II
FRACTIONATION OF *Escherichia coli*

Equivalent of 930 mg. dry wt. organisms disrupted mechanically in standard saline (0.17 M NaCl, 0.017 M KCl, 0.005 M MgCl₂) for 30 min. using the disintegrator of H. Mickle as described by Mitchell and Moyle [27], washed into centrifuge tubes with c. 30 ml. standard saline at 2 and centrifuged at 2000 g for 30 min.

	↓		↓
	Supernatant centrifuged at 35 000 g for 1 hr.		Pad in 2 layers: Top, P ₁ (5 ml.). Bottom, P ₂ (1 ml.).
	↓		↓
Supernatant, S ₁ (48 ml.).	Pad in layer, P ₃ (2 ml.). Dispersed in 10 ml. standard saline. Morphology: aggregates of very small particles.	P ₁ , easily dispersed, made up to 10 ml. in standard saline. Morphology: empty envelopes, intact and fragmented.	P ₂ , dispersed with difficulty, made up to 10 ml. in standard saline. Extinction at 700 mμ equivalent to 93 mg. dry wt. intact cells. Morphology: mainly intact cells.
			↓
			Of fraction P ₁ , 8 ml. returned to sonic disintegrator for 15 min. Washed into centrifuge tubes with standard saline (total vol. 14 ml.). Centrifuged at 2000 g for 60 min.
		↓	
		Supernatant, S ₂ (12 ml.).	Pad, P ₄ (2 ml.). Made up to 8 ml. in standard saline. Morphology: as P ₁ , but more fragmented.

The morphology of the fractions was examined by anoptical contrast microscopy of very thin films of the untreated aqueous suspensions sealed between slide and coverslip with a ring of vaseline.

such "soluble", extracellular enzymes—like β-galactosidase in *Escherichia coli* [40, 41]—cannot be involved in catalytic activity at the surface of the protoplast, or in membrane transport, because they are said to be "cryptic", or, in other words, enclosed behind the osmotic barrier component of the plasma membrane [1]. Dr. Stephen and I have studied the "solubility" and distribution of glucose-6-phosphatase activity in *Escherichia coli*

(strain ML 30) with these assumptions in mind. We found, as illustrated in Tables II and III, that after disintegrating washed suspensions of *Escherichia coli* mechanically, and fractionating on the centrifuge, some 85% of the glucose-6-phosphatase was present in the clear solution that had been centrifuged at 35 000 g for 1 hr., and some 12% was initially present in the cell envelope fraction which centrifuged down at 2000 g in 30 min. On re-submitting the cell envelope fraction to the disintegration procedure, to release any enzyme that might have been trapped by reclosure of some of the membranes, a further 5% of the enzyme was obtained in the "soluble" form, bringing the amount of "soluble" glucose 6-phosphatase recovered to some 90% of the whole. These and other related

TABLE III

DISTRIBUTION OF GLUCOSE 6-PHOSPHATASE IN *Escherichia coli* (ML 30)

Fraction	Glucose 6-phosphatase activity (μ mole P/g. min.)	% Total activity
<i>From whole cells</i>		
"Soluble", S ₁	1.80	84.7
Very small particles, P ₃	0.064	3.0
Cell envelopes, P ₁	0.263	12.3
<i>From redisintegrated cell envelopes</i>		
"Soluble", S ₂	0.098	4.6
Cell envelopes, P ₄	0.128	6.0
<i>Intact untreated cells</i>	2.11	99

experiments showed that although the glucose-6-phosphatase probably has an affinity for a cell envelope component, according to the usual standards it would be classed as a soluble enzyme. We discovered, however, as illustrated at the bottom of Table III, that the rate of hydrolysis of externally added glucose-6-phosphate by suspensions of intact cells represents the full expression of the "soluble" enzyme activity, and, as shown in Table IV, the activity of the intact cells was little affected by breaking the plasma membrane with benzene (5% v./v.) or by freezing and thawing. We showed that glucose-6-phosphate does not penetrate into the protoplast of intact cells, for although it could be fermented rapidly by cells in which the membrane was ruptured, in intact cells it was fermented only at a rate corresponding to that of the liberation of free glucose by the fully expressed glucose-6-phosphatase. Further, the glucose-6-phosphatase of intact cell suspensions was found to liberate the inorganic phosphate of externally added glucose-6-phosphate, not in the protoplasts, but in the

TABLE IV

GLUCOSE 6-PHOSPHATASE ACTIVITY OF NORMAL AND TREATED *Escherichia coli* (ML 30) SUSPENSIONS AND OF GROWTH AND SUSPENSION MEDIA

Material	Glucose 6-phosphatase activity (μ mole P/g. min.)	% Activity of normal cells
Normal intact cells	2.39	100
Benzene-treated cells	2.77	116
Frozen and thawed cells	2.19	92
Suspension medium	0.11	4.6
Growth medium	0.07	2.9
	(μ mole P/g. min. liberated in suspension medium only)	
Normal intact cells	2.91	122

suspension medium. As illustrated in Fig. 1, these and other confirmatory observations forced us to the conclusion that the glucose-6-phosphatase of intact *Escherichia coli* is enclosed in a region between the cell wall and the surface of the osmotic barrier component of the plasma membrane which

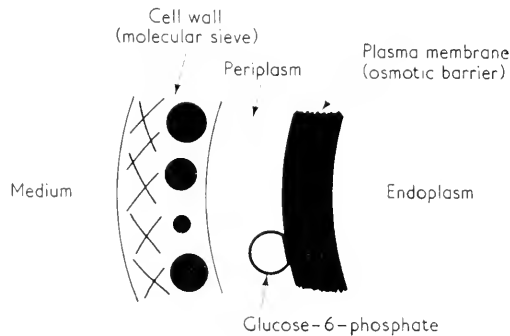


FIG. 1. Diagram of cell wall, periplasm and plasma membrane (osmotic barrier component) in *Escherichia coli*. The glucose-6-phosphatase, partly adsorbed on a substratum in the cell envelope complex, is confined to the periplasm by the molecular sieve function of the cell wall.

we might appropriately call the "periplasm". You may ask how one can show that the effective pore size of the cell wall of living *Escherichia coli* is small enough to prevent the passage of proteins between the periplasm and the external medium. Figure 2(a) shows living *Escherichia coli* (strain ML 30) in which the protoplasts have been made to retract from the cell wall by the addition of 0.4 M NaCl to a suspension medium of 0.02 M sodium phosphate buffer at pH 7, and Fig. 2(b) shows the same with the

further addition of 15% w./v. human serum albumin. The brightness of the anoptical contrast image increases with the refractive index of the object. If the serum albumin had penetrated into the periplasm, enlarged by plasmolysis, there would be no more contrast between the periplasm and the suspension medium in Fig. 2(b) than in Fig. 2(a). The fact that the periplasm is darker than the suspension medium in Fig. 2(b) shows

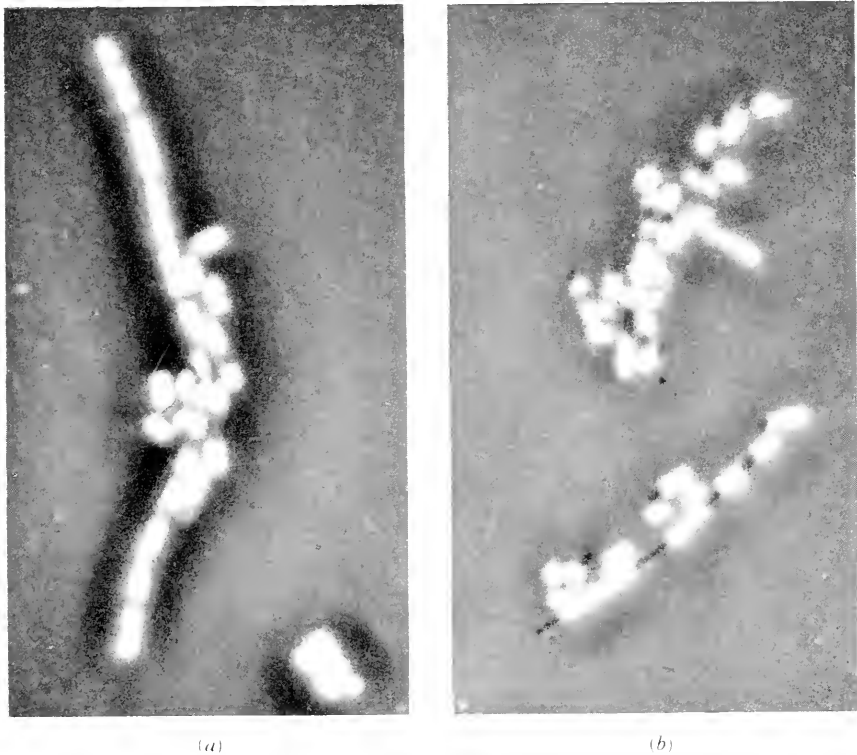


FIG. 2. Anoptical contrast micrographs ($\times 3800$) of *Escherichia coli* (ML 30) plasmolyzed in 0.4 M NaCl (a), and the same with addition of 15% (w./v.) serum albumin (b). The dark periplasm between cell wall and retracted protoplasts in (b) shows impermeability of cell wall to serum albumin.

that the serum albumin (M.W. 70 000) does not penetrate the cell wall. It seems reasonable to conclude that the cell wall acts as a molecular sieve, allowing entry of glucose 6-phosphate, but preventing the escape of glucose 6-phosphatase from the surface of the protoplast. The glucose 6-phosphatase is thus in a position to catalyze the first step in the metabolism and uptake of glucose 6-phosphate in *Escherichia coli*.

The facts established by this work have far-reaching implications for

the interpretation of observations on enzyme distribution in relation to membrane transport in micro-organisms. When we conceive the cell wall as a molecular sieve, preventing the loss of enzymes that may exist entirely, or only partly, in the free state at the surface of the plasma membrane (i.e. in the periplasm), we must give serious consideration to the possibility that the enzymes and catalytic carriers of the protoplasm may be poised in an equilibrium which may favour their segregation in the periplasm, in the plasma membrane, or in the endoplasm according to the satisfaction of mutual affinities. The distribution of a given enzyme, and its status as a relatively "soluble" or "insoluble" protein would thus be seen as an expression of locational affinities for bonding the protein to complementary substratum sites in the cell. In general, it would be expected that such bonding would be due to secondary valencies, but it is also possible that primary valencies might sometimes be involved. The studies of Keilin and King [42] on the reversible bonding of the soluble succinic dehydrogenase in the insoluble cytochrome system of heart muscle lends support to this conception. Such a conception has obvious potentialities for helping to explain induction and repression of enzyme synthesis by a mass action type of effect [43, 44] not only thought of as being due to equilibration of nascent enzyme with enzyme-substrate or enzyme-(substrate analogue) complexes, but also due to the equilibration of nascent enzyme with enzyme-substratum and (enzyme-substrate)-substratum complexes. These attractive ideas, which are related to those of Catcheside [45], hinge, however, on the experimental demonstration of the locational affinities between enzymes and substratum sites in bacteria. For reasons that I shall explain in a moment, Dr. Moyle and I decided to study the distribution of the α -ketoglutarate dehydrogenase activity in *Micrococcus lysodeikticus* as an example of the possible participation of locational affinities in determining the cytological distribution of an enzyme.

When *Micrococcus lysodeikticus* is ruptured by shaking with glass beads or by gentler enzymic and osmotic methods, and the plasma membranes are separated from the "soluble" or "protoplasm" fraction on the centrifuge in the usual way [see 11, 30], about half the α -ketoglutarate dehydrogenase activity is found in the plasma membrane fraction. The amount of enzyme activity attached to the plasma membranes is not dependent upon the distribution of dialyzable cofactors, nor is it appreciably affected by the extent to which the membrane material is diluted during its isolation. One can therefore infer that the enzyme must be strongly bound to the membrane structure. The fact that, nevertheless, about half the enzyme activity is present in the "soluble" fraction shows either that there are two α -ketoglutarate dehydrogenase proteins with different solubilities or affinities for membrane and protoplasm components, or that there is only one type of α -ketoglutarate dehydrogenase, which is a soluble protein, and

that the membrane contains a fixed number of substratum sites at which this soluble enzyme can be specifically bonded. We designed a series of experiments to decide between these alternatives, and I can best indicate briefly our conclusion by the example from part of an experiment shown in Table IV. The method of approach was to inactivate the membrane-located enzyme in the intact cell with an irreversible inhibitor (in this case, iodoacetate at pH 8), and then to determine whether the enzyme could be replaced *in vitro* by the "soluble" enzyme of the normal protoplasm

TABLE V

EQUILIBRATION OF α -KETOGLUTARATE DEHYDROGENASE BETWEEN THE MEMBRANE AND PROTOPLASM OF *Micrococcus lysodeikticus*

	Membrane	"Protoplasm"
Normal cells	55.5	44.5
Iodoacetate-treated cells	14.6	4.5
Iodoacetate-treated membrane (1)	14.6	—
Normal "Protoplasm" (2)	—	40.0
After re-separating mixture of (1) and (2)	35.5	20.0

fraction. The numbers in Table V represent α -ketoglutarate dehydrogenase activity expressed as a percentage of the total activity of the normal intact cells, which, in this experiment, had an absolute value of $0.65 \mu\text{mole}$ substrate per g. cell dry weight per minute. Rather more than half the total activity was initially present in the membrane fraction in this batch of cells. After reacting the intact cells with iodoacetate and separating the membrane and "protoplasm" fractions as usual, the total α -ketoglutarate dehydrogenase activity was reduced to about 20% of the normal. A sample of the inactivated membrane fraction, representing an activity of 14.6, was now thoroughly mixed with a sample of the normal "protoplasm" fraction, representing an activity of 40, and the two fractions were separated again on the centrifuge. As shown in Table V, the activity of the membrane fraction was found to have risen by 20.9 units, while that of the "protoplasm" had fallen by 20.0 units—an equivalent amount within experimental error—showing a substantial transfer of enzyme from the "soluble" state in the "protoplasm" fraction to the bound state in the membrane complex. This, and other confirmatory and related experiments imply that the distribution of α -ketoglutarate dehydrogenase activity in *Micrococcus lysodeikticus* does indeed depend upon the mutual satisfaction of locational affinities between a soluble α -ketoglutarate dehydrogenase and a specific substratum in the plasma membrane complex of the cell.

Special characteristics of vectorial metabolism in anisotropic enzyme systems

The studies that Dr. Moyle and I have recently been carrying out on the specificity and general kinetics of the entry of " α -ketoglutarate" and "succinate" into *Micrococcus lysodeikticus*, and the comparison of these characteristics with those of the α -ketoglutarate dehydrogenase, succinic dehydrogenase, succinyl-coenzyme A kinosynthetase, and other enzymes present in the plasma membrane complex ([19, 46], and extensive unpublished observations) has led us to represent the entry mechanism by the tentative scheme of Fig. 3. This scheme is in accord with all the experimental facts at present available to us, but I must emphasize that it is

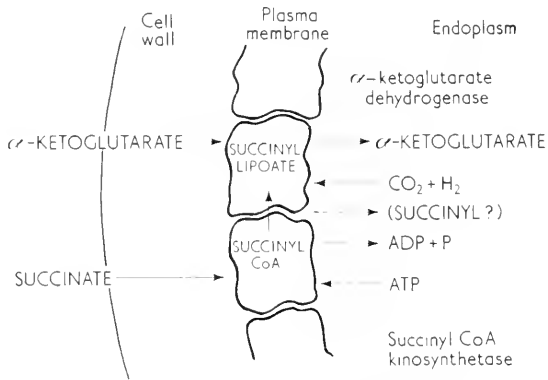


FIG. 3. Diagram of "succinate" and " α -ketoglutarate" translocation through the membrane of *Micrococcus lysodeikticus*. The dehydrogenases are depicted as part of the plasma membrane, anchored to each other (and to other substrata not drawn) by specific residual bonds.

nevertheless tentative; for, when one considers how unequivocal the interpretation of such observations can be made, or in other words, how close a correspondence one would expect to find between the various constants (Michaelis constants, inhibitor constants, pH characteristics, temperature coefficients, etc.) measured on the one hand for the intact membrane system and on the other hand for the isolated enzymes and carriers, it is apparent that not only the kinetic "constants" but also the apparent thermodynamic "constants" of the reactions can be profoundly affected by the anisotropic situation of the catalysts in the membrane. It will, I think, be appropriate to conclude my paper with a brief discussion of this aspect of translocation catalysis, for it has an important bearing on any experiments designed to identify the catalysts of membrane transport by comparing their kinetic and thermodynamic constants *in situ* in a

membrane or other complex with the "corresponding" constants determined in homogeneous solution for the soluble enzymes or catalytic carriers extracted from the complex. The train of thought that I propose to follow will incidentally suggest a new way in which the function of the particulate systems that couple oxidoreductive or photon-activated electron transport to phosphorylation could be dependent upon their structure. But I do not propose to do more than touch upon this incidental suggestion today as it would lead us outside the context of this session of the Symposium.

SOME EFFECTS OF ANISOTROPY UPON AN ENZYME-CATALYZED HYDROLYTIC REACTION

For the sake of simplicity, let us consider the effect of one variable, pH, on an enzyme catalyzed reaction of the hydrolase type represented by the equation,



Kinetic considerations

When the enzyme, E, is dissolved in a homogeneous aqueous medium, the usual constants, such as the maximum velocity or the Michaelis constant, will vary with the pH of the medium, and the kinetic constants of this variation will be characteristic of the enzyme because the catalytic activity depends upon the degree of ionization of acidic and basic groups in different parts of the protein molecule. If, however, the enzyme is situated in a membrane separating two aqueous phases which are poised at different hydrogen ion activities, the degree of ionization of the acidic and basic groups on the two sides of the enzyme will be different, and as the state of the enzyme molecules will not be defined by a single pH value, we cannot properly define the kinetic constants relating the characteristics of the enzyme activity to the pH. It follows from the generalization of this kind of consideration, that the kinetic constants of enzymes or catalytic carriers as usually defined in homogeneous systems are not strictly comparable to the "corresponding" constants for the same catalysts when present in natural membranes or other anisotropic complexes.

Thermodynamic considerations: Chemiosmotic coupling

In the homogeneous system represented by equation (1), the enzyme will catalyze the equilibration of the reaction according to the equation,

$$\frac{[AH] \times [BOH]}{[AB]} = K' \quad (2)$$

in which the square brackets stand for electrochemical activities and K' is a thermodynamic "constant", independent of the properties of the enzyme and independent of pH, other things being equal. In writing equation (2), I have followed the custom of omitting the activity of the water in the system as this is a constant in homogeneous aqueous physiological media, and has a value corresponding to 55.5 M water. In an inhomogeneous (pseudo-equilibrium) system, however, the activity of the water at the site (ϵ) of the hydrolytic process may not correspond to that of physiological aqueous media, and in this case it must be included as follows,

$$\frac{[\text{AH}]_{\epsilon} \times [\text{BOH}]_{\epsilon}}{[\text{AB}]_{\epsilon} \times [\text{H}_2\text{O}]_{\epsilon}} = K' \quad (3a)$$

$$\frac{[\text{AH}]_{\epsilon} \times [\text{BOH}]_{\epsilon}}{[\text{AB}]_{\epsilon}} = K'[\text{H}_2\text{O}]_{\epsilon} \quad (3b)$$

It will be seen that the usual hydrolysis "constant", K' , is a variable which is proportional to $[\text{H}_2\text{O}]_{\epsilon}$, the electrochemical activity of the water at the active centre of the enzyme.

It is a well-known fact that enzyme reactions—such as the catalysis of the transfer of the phosphoryl group (or phosphorylium ion) by phosphokinases—can be effectively anhydrous even though the enzyme molecules are surrounded by water. It would not, therefore, be unrealistic to assume that the active centre of a hydrolytic enzyme, situated anisotropically in a membrane complex, could be inaccessible to water, but could be accessible to hydrogen (but not hydroxyl) ions from one side (phase I) and could be accessible to hydroxyl (but not hydrogen) ions from the other side (phase II). For the sake of simplicity, we will not make any assumptions about the anisotropy of the enzyme with respect to the accessibility of AB, AH, and BOH to its active centre. The electrochemical activity of the water at the active centre, ϵ , of the enzyme would be given by the dissociation constant of the water, K_w , defined as follows,

$$[\text{H}_2\text{O}]_{\epsilon} = \frac{[\text{H}^+]_{\text{I}} \times [\text{OH}^-]_{\text{II}}}{K_w} \quad (4)$$

$$\text{and since, } [\text{OH}^-]_{\text{II}} = K_w \frac{[\text{H}_2\text{O}]_{\text{I or II}}}{[\text{H}^+]_{\text{II}}} \quad (5)$$

equation (4) can be written,

$$[\text{H}_2\text{O}]_{\epsilon} = \frac{[\text{H}^+]_{\text{I}} \times [\text{H}_2\text{O}]_{\text{I or II}}}{[\text{H}^+]_{\text{II}}} \quad (6)$$

The effect of the anisotropic situation of the hydrolytic enzyme on the dissociation equilibrium represented by K' can consequently be expressed as follows, using (3*b*) and (6),

$$K' = \frac{[\text{AH}] \times [\text{BOH}]}{[\text{AB}]} = K[\text{H}_2\text{O}]_{\text{I or II}} \times \frac{[\text{H}^+]_{\text{I}}}{[\text{H}^+]_{\text{II}}} \quad (7)$$

This equation shows that the poise of the dissociation equilibrium represented by K' is proportional to the ratio of the hydrogen ion electrochemical activity in phase I to that in phase II. The electrochemical activity of the hydrogen ion in the two phases may, of course, differ either because of a difference of hydrogen ion chemical activity or because of a membrane potential, a potential of about 60 mV being equivalent to a hydrogen ion chemical activity ratio of 10. The membrane potential or hydrogen ion chemical activity difference across the membrane could be generated by a photoelectric effect or by a metabolic oxidoreduction involving a flow of electrons across the membrane. Equation (7) shows that the work done in creating the asymmetry of $[\text{H}^+]$ across the membrane can be coupled to synthesis of AB by dehydration of AH and BOH, the hydrogen ions of the water that is eliminated travelling to phase I and the hydroxyl ions travelling to phase II. Synthesis of AB is, of course, promoted by lowering the chemical activity of the hydrogen ion or by a negative potential in phase I relative to phase II.

It will be helpful to consider the reaction catalyzed by glucose-6-phosphatase as a relevant and quantitative example of the above principle of chemiosmotic coupling. The equilibrium constant, K' , for the hydrolysis of glucose-6-phosphate to glucose and inorganic phosphate is approximately 250 in homogeneous aqueous solution at pH 7 [47], and the concentration of glucose-6-phosphate in equilibrium with 10^{-2} M glucose and 10^{-2} M phosphate would be only 4×10^{-7} M. If glucose-6-phosphatase were located in the anisotropic membrane complex as described above, a pH difference of only 3 units between phases I and II, or a potential difference of 60 mV and a pH difference of 2 units, would lower the dissociation constant by a factor of 1000 and would raise the concentration of glucose-6-phosphate in equilibrium with 10^{-2} M glucose and 10^{-2} M phosphate to 4×10^{-4} M—a concentration high enough to enter the phosphohexose isomerase reaction of the glycolytic pathway at near the maximum rate. This fact is all the more interesting since the glucose-6-phosphatase of *Escherichia coli* (as discussed above) and of liver cells [48] appears to be appropriately situated in a membrane complex. I need hardly point out that a similar, but greater asymmetry of electrochemical hydrogen ion activity to that considered in the above example, could be responsible for converting the ATPases of the particulate systems of photosynthetic and oxidative phosphorylation into the ATP-synthesizing

catalysts. I hope to develop this interesting and important aspect of translocation catalysis on another occasion.

My purpose in concluding with these rather brief thoughts on what I have called vectorial metabolism was two-fold. First, these thoughts add something to our conception of the intimate relationship between transport and metabolism; and second, they pose a most important experimental question. The activities of the translocation catalysts in their natural situation in membranes or other anisotropic complexes are not strictly comparable to their activities in the homogeneous solutions in which we are accustomed to isolate and study them. How, then, can we proceed to identify the translocation catalysts and demonstrate the molecular mechanism of their activity? I believe that the only satisfactory answer to this question is to be found in the fourth method of approach to the analysis of membrane transport that I mentioned at the beginning of this paper. We must strive to set up "synthetic" or reconstituted membrane systems with which we can study directly both the processes of transfer (in the normal biochemical sense) and the processes of translocation, catalyzed by enzymes and catalytic carriers under anisotropic conditions that can be controlled and measured.

I am indebted to Dr. Jennifer Moyle for helpful general discussions during the preparation of this paper, to Dr. J. Dainty for help in describing the electrochemical activity in relation to escaping tendency, and to Dr. P. H. Tuft for suggesting the word "substratum" as a synonym for enzyme-locator. I am also glad to acknowledge grants from the Nuffield Foundation in aid of this work.

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Discussion

DISCIE: Is this substance which is responsible for permeability and for changes in permeability really an enzyme? The evidence based on such phenomena like competitive inhibitions seems completely consistent with the idea that the membrane structure depends on properties of certain proteins in the membrane and for the phenomena which are very familiar about binding of certain substances to the protein, and the competitive inhibition of penetration of substrates by analogous substances, would be adequately explained by specific binding of these substances

by proteins. The concept of an enzyme implies that the substance which penetrates is chemically changed and I should like to ask if changes in penetrating substances occur?

MITCHELL: Well, Dr. Dische, you are asking rather a big question. Let me answer first of all with an example that stems from my paper. In the utilization of glucose 6-phosphate by *Bacterium coli*, what actually passes through the membrane is, on the one hand "glucose", and on the other hand "phosphate". We know this because the rate of utilization of glucose 6-phosphate is the same as the rate at which the externally available glucose 6-phosphatase can break it down. This is a clear example of a case in which the first process in the overall transport reaction is an enzymic one. We have to be very careful in speaking, for example, of the transport of glucose 6-phosphate into the cell, because we know that the glucose 6-phosphate does not go in, although later glucose 6-phosphate is found in the cell as a result of the separate entry of the "glucose" and the "phosphate" by different molecular pathways. Moreover, there is evidence that the membrane is impermeable to glucose and phosphate and that these molecules pass across the membrane as derivatives or chemical groups (hence the inverted commas). This illustrates a general principle that I am trying to make, but I do agree with you, that we must not jump to silly conclusions; we must not say that we have direct evidence for the process of group translocation, and as I pointed out in my paper, this evidence may well have to await the successful reconstruction of *in vitro* membrane systems. All the same, the specificity and kinetics, and especially the susceptibility to inhibitors, of a number of transport processes do strongly suggest that they represent enzyme-catalyzed chemical reactions. Let us consider, for example, the entry of "succinate" into micrococci. In this case we can examine the catalytic system in rather an elegant way. Some years ago, Dr. Moyle and I thought that the entry might be directly through succinic oxidase, and we argued that as succinic oxidase is part of the membrane it could have its active centre exposed on the outside. As we knew that the membrane is impermeable to succinate and malonate in the normal sense, we were able to investigate this possibility by seeing whether external malonate would inhibit the succinic oxidase of intact cells. In fact, malonate was found to have no effect at all unless at first you depressed the pH to 5 to let the malonate in and then brought it back to 7. After that the external malonate could be washed away, and succinate oxidation continued to be inhibited. This shows that the succinic oxidase is inhibitable by malonate, but the active centre is facing inwards. It also shows that the specificity of the process giving rise to "succinate" entry is such as to discriminate between malonate and succinate. This is one of the reasons why we think that the kinosynthetase may catalyze the first reaction for succinate entry, for this enzyme, unlike succinic oxidase, does distinguish between succinate and malonate and is not inhibited by the latter. Further, the substance that passes into the cytoplasm of the micrococcus while succinate passes into the outer surface of the plasma membrane is not succinate! This illustrates the background of my approach to the closely related problems of transport and metabolism in whole cells.

In kinetic studies, the evidence obtained is circumstantial. One creates hypotheses in order to disprove them. Our aim has been in the past to try to

formulate hypotheses in accord with as much of the circumstantial evidence as possible, and I think that our aim now must be to try also to develop *in vitro* membrane systems in which these hypotheses can be put to more crucial tests.

FRENKEL: In the scheme which you showed on your slide glucose 6-phosphatase appears to be present in the cell wall and it is thus difficult to see how it can be solubilized readily.

MITCHELL: I did not say that it was in the cell wall. What I said was that I believed it must be present in the space between the inner margin of the wall, which we know to be impermeable to proteins, and the outer limit of the osmotic barrier component of the plasma membrane. I agree, I think, with the implication of Dr. Frenkel's remark—that we have to be a little careful about the words describing the cytological structures when we get to such molecular dimensions. The plasma membrane is quite thick—thick enough to be regarded in some respects as a separate phase. There is one functional component of the plasma membrane (we are not sure whether it is in the middle, or near its outer or inner surface), which is mostly a hydrophobic sheet, part probably being protein and part lipid. This hydrophobic sheet is called the osmotic barrier. If we find a catalytic activity exhibited only on the outside of this, the active centre of the catalyst responsible for that activity must be situated somewhere outside the osmotic barrier, or must be in a crevice accessible to the substrate only from the outside. Now, the situation could be that the glucose 6-phosphatase is tucked into or attached to the osmotic barrier, its active centre being accessible to glucose 6-phosphate from outside. When you break the cell, because of the great changes in ionic environment, etc., the enzyme might well become dissociated from the membrane complex and appear as a soluble protein—just as we find it.

DORFMAN: I wonder if we are not prisoners of our conventional definitions of enzymes and specific proteins. I am thinking of what Dr. Davis said about the analogy of a permease and haemoglobins. In a sense when oxygen is bound it is chemically changed but it is released as the same substance; in the same way as the transport of glucose as glucose 6-phosphate might occur. I wonder whether we shouldn't think more in terms of protein specificity and less of an enzyme as a catalyst which must bring about a chemical change in the more conventional organic chemical sense?

MITCHELL: I agree with that remark, but I take exception to the suggestion that we are being blinded by a conventional and old attitude towards enzymes. After all, enzyme kinetics is a growing subject. I agree with Dr. Dorfman that we need to try to obtain a more biochemical view of transport processes, but I use the word biochemical to mean conceived in the most up-to-date organic and physical chemical terms. I have no preconceptions as to whether the catalysts of molecule, ion, group, and electron translocation will turn out to be enzymes in the sense that may currently be in use. But I would point out that in the enzyme field we have a number of catalytic carriers, such as the flavoproteins and the haem proteins of the cytochrome system, and one would not say that the cytochrome system caused a chemical change in the electrons which passed through it. Nevertheless, we generally regard these catalysts as part of the overall enzyme system. It is in this sort of context that I am trying to speak. I suppose that if we found proteins with quite new capacities, which were unlike haemoglobin, unlike the catalysts of the

cytochrome system, and unlike the proteins universally accepted as enzymes, then we would be justified in inventing a new name to describe them. But I would hesitate to consider such special proteins until we have isolated at least one.

DAVIS: I would like to ask Dr. Porter to comment on another aspect of this problem. Dr. Mitchell has stressed the advantages of bacteria arising from their smallness and simplicity, but of course we are all aware of disadvantages which also arise from smallness, such as the difficulty of recognizing morphological sub-units. Now electron microscopists until very recently have all agreed that there is no endoplasmic reticulum in bacteria; but a few months ago Glauert published in the *Journal of Biochemical and Biophysical Cytology* some pictures showing with new methods of fixation what appears to be a very fine reticulum in parallel lamellae in an actomycete (which is fundamentally an elongated bacterium). I wonder whether Dr. Porter would care to comment on the generalization that bacteria may or may not have such a reticulum.

PORTER: Audrey Glauert at the Strangeways Laboratories in Cambridge, England, has taken and published very informative micrographs of complex membrane systems in *Streptomyces* and some suggestion has been made that these may be analagous to the endoplasmic reticulum of other cells. Actually the bacterial cytoplasmic membranes seem to be infoldings of the membrane limiting the protoplast and could represent an attempt on the part of the cell to increase a surface available to diffusible metabolites. Certain blue-green algae show a similar complex infolding of the plasma membrane. They are evidently common to lower forms which do not possess a nuclear envelope and associated ER. Whether such complex infoldings of the surface membrane have evolved into the surface-independent endoplasmic reticulum of higher cell forms is an interesting topic for speculation.

DAVIS: Well, I wonder, Dr. Mitchell, whether you have any evidence as to whether or not certain activities which you observed in the centrifugable fraction, particularly those of the cytochrome system and the TCA cycle, could be in small particles attached to the membrane rather than in the substance of the membrane itself.

MITCHELL: Yes, I think this query is a very difficult thing to resolve experimentally. It is also difficult to speak about. The succinic dehydrogenase active centre is certainly inside the osmotic barrier. If you think of the succinic oxidase as a particle, you may imagine that this piece of the cytochrome, as well as the succinic dehydrogenase attached to it, is under the osmotic barrier. You may, if you are a biochemist, regard the plasma membrane as a hydrophobic sheet with various activities attached to it as particles, which can be isolated and characterized biochemically. But, if you are a cytologist, you will be impressed by the fact that under certain conditions the plasma membrane complex behaves as a single mechanical unit containing the enzyme activities of all its so-called particulate constituents. I think that the work in Dr. Weibull's laboratory and in my own laboratory has now established this fact in the case of *Bacillus megaterium*, *Staphylococcus aureus* and *Micrococcus lysodeikticus* beyond any reasonable doubt. One would presume that the residual bonding that is holding the various parts of the membrane complex system together is stronger in some places than in others; so that if you treat it very kindly, as you must do if you wish to isolate cytologically

recognizable membranes, it may remain more or less intact, but rougher treatment makes it fall to pieces. Standing midway between the cytologist and the biochemist, we may presume that the "pieces" (of the cytologist) are the "particles" or groups of "particles" (of the biochemist). I think that we need to become as interested in the substratum (or locational) specificities of enzymes as we have been in their substrate specificities; and the elegant work on mitochondrial structure which Dr. Lehninger described earlier in this symposium is clearly moving in this direction. To take the analysis of the jig-saw organization of the plasma membrane and other membrane complexes further will require a great collaborative effort between biochemist and cytologist. Perhaps one of the most important problems to settle at the present stage is whether there is a general substratum substance which represents the mechanical matrix of the membrane and acts as the locator for the enzymes and carrier proteins, or whether the individual molecules of the enzymes, carriers, lipids and other components of the complex represent the substrata for each other and share the responsibility for structural (locational) and catalytic properties of the membrane fabric.

This is a very difficult problem, and as I have already pointed out, it may well be impossible to make real progress until we have learned to study the material of natural membranes in anisotropic *in vitro* systems. Perhaps Dr. Albertsson's polymer systems would be helpful for this purpose. We discussed this privately the other day, for it struck me that the anisotropic properties of the interface between the two aqueous polymer phases might be varied at will over quite a wide range. Such a system might prove to be useful for studying the behaviour of biologically active molecules under anisotropic conditions, and might serve as a starting point for setting up reconstituted membrane systems *in vitro*.



Protein Uptake by Pinocytosis in Amoebae: Studies on Ferritin and Methylated Ferritin*

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It has been suggested that pinocytosis may be the underlying process in many transport phenomena. The idea has been reviewed by Dr. Holter, in this Symposium and elsewhere [1]. Such a view seems to have been especially attractive to electron microscopists, who have found evidence for vesicle formation in a variety of cells. Physiologists, however, who have studied and defined active transport by other methods, have felt that the engulfment of droplets of the cell's environment is too indiscriminate a process to account for the highly specific effects which, as Dr. Davis has pointed out in his review for this Symposium, are characteristic of active transport. There are other equally serious inadequacies in the notion that pinocytosis is simply the morphological equivalent of active transport.

The chief difficulty in this debate is a familiar one; so long as we do not understand the mechanisms of pinocytosis, on the one hand, or of active transport, on the other, we are free to launch hypotheses which can soar quite freely. The purpose of this communication is therefore to describe experiments which were designed to answer two questions about protein uptake by pinocytosis in amoebae. First, what is the physical mechanism of protein binding to the cell surface, the binding which is known to set off the pinocytosis response? And second, what happens to the pinocytosis vesicle, and to its contents, after it is taken into the cell? Note that these questions concern only the first and last stages of the pinocytosis response; between these there occurs the actual process of invagination, the formation of tunnels and vesicles. This must also be studied experimentally, but it will be seen from the results that the first and last stages are those most directly related to the problem of transport between the environment and the cytoplasm.

The studies of Schumaker [2] and of Brandt [3] supplied the first evidence that protein uptake by amoebae began with some type of binding

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reaction to the cell surface, and was not, as had been assumed previously, a simple matter of the cell engulfing droplets of the medium. Schumaker's kinetic studies showed also that the binding reaction was not affected by cooling or by metabolic inhibitors, although the later stages of vesicle formation were readily blocked. Additional evidence from several sources suggested that the mechanism of the binding reaction was electrostatic [4, 5, 6] and that the receptor substance was the mucous coat which covers the amoeba. In the work here reported, the mechanism of the reaction was studied by comparing the pH dependence of binding for two closely related proteins, ferritin and methylated ferritin. Binding studies were done on living amoebae (*Chaos chaos* or *Pelomyxa carolinensis*) and the results were confirmed by electron microscopy. The same proteins were used in further studies on the changes which occur within the cell after uptake.

Methods

At the outset, it was found that protein binding by the cell surface could be "uncoupled" from the remaining stages of pinocytosis by working at 5° [2]. Starving amoebae were rinsed in cold water. By this treatment, the cells were rounded up, cytoplasmic motion was suppressed, and contaminating ions were removed. The amoebae were then pipetted into the cold protein solution, left for 3 to 5 min., and washed in the cold to remove all unbound protein. The washing procedure made it possible to test the reversibility of the surface binding at different pH values [5]. Some cells were fixed at this point in buffered osmium tetroxide, and were embedded and sectioned in epoxy resin for electron microscopy. Others were allowed to warm to room temperature; in such cells, the complete pinocytosis sequence occurred despite the delay, and the protein carried into the pinocytosis vesicles was only that previously bound to the cell surface and not removed by washing. As a result, it was possible to follow the changes which occurred within the vesicles more clearly than when "bulk" pinocytosis was induced at room temperature. Cells treated in this way were kept in their normal medium for 1 to 48 hr. after uptake, and were then fixed, embedded, and sectioned.

PROPERTIES OF FERRITIN AND METHYLATED FERRITIN

Ferritin was isolated from horse spleen by ammonium sulphate precipitation followed by crystallization in the presence of cadmium sulphate [7, 8]. It has some unusual properties [9, 10], which made it especially suitable for this study. The unit particle of ferritin, which is 94 Å in diameter, consists of a protein coat surrounding an ordered cluster

of micelles of ferric hydroxide and ferric phosphate. The particles are of uniform size and shape, and the internal cluster of dense iron micelles makes it possible to identify in high resolution electron micrographs even a single particle [11, 12]. The protein coat is responsible for the electrochemical properties of ferritin, such as electrophoretic mobility and solubility. Ferritin behaves, therefore, as a typical protein ampholyte, with an isoelectric point of 4.4 [13].

A methylated derivative of ferritin was prepared by esterifying the protein in acid methanol [14]. The effect of methylation was judged by comparing at several pH values the solubility of the product with that of the original protein. Solubility (in optical density units) was measured by determining the absorbance at 280 m μ of supernatant solutions in equilibrium with precipitated proteins (Fig. 1).

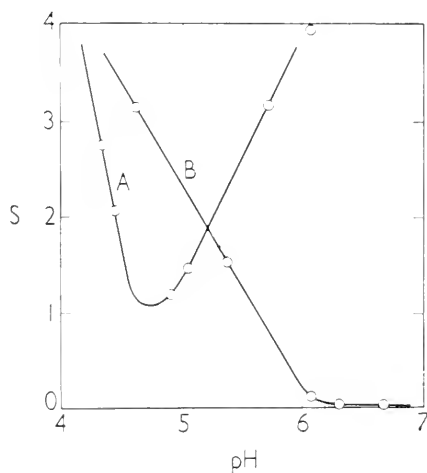


FIG. 1. Solubility of ferritin (curve A) and of methylated ferritin (curve B), as a function of pH. Ordinate: solubility in optical density units, at 280 m μ .

No direct measure of the percentage of carboxyl groups blocked was attempted, but the solubility curves demonstrated that the charge properties were significantly modified by methylation. The solubility of normal ferritin above pH 5 is almost entirely due to the ionization of carboxyl groups. When these are blocked by extensive methylation, the solubility at pH 6 to 7 drops to a very low value (about 10⁻⁴%, by weight). In electron micrographs, the unit particles of methyl ferritin appeared the same as those of ferritin. It seemed safe to conclude that methylation did not grossly alter the structure of the ferritin particle, and that any difference in binding or uptake by amoebae could be correlated with the specific charge effect of blocking carboxyl groups.

EFFECTS OF pH ON BINDING

The effects of pH on the binding of ferritin and of methyl ferritin to the cell surface were studied. Because the proteins had an intense orange colour, the results of binding and washing experiments could be followed by direct observation of the living cells. The results were confirmed by electron microscopy.

At pH 4, when both proteins were positively charged, both were bound, and both invoked the pinocytosis response. At neutral or slightly acid pH,



FIG. 2. Binding of ferritin to surface coat of amoeba *Chaos chaos*, at pH 4. The binding persists after washing at pH 4 for 5 to 10 min.

when its particles carried a net negative charge, ferritin was not bound and did not invoke the pinocytosis response.

Methyl ferritin was too insoluble at neutral pH to permit a direct comparison of its binding with that of ferritin, but a clear difference in the reversibility of binding was seen in washing experiments. Once bound to the cell surface at pH 4, neither protein was removed by washing in the cold at pH 4. When the washing was done above pH 5, ferritin was quickly removed, but methyl ferritin was not.

Figure 2 is an electron micrograph which shows ferritin bound to the cell at pH 4, and remaining bound after washing at the same pH. Note that

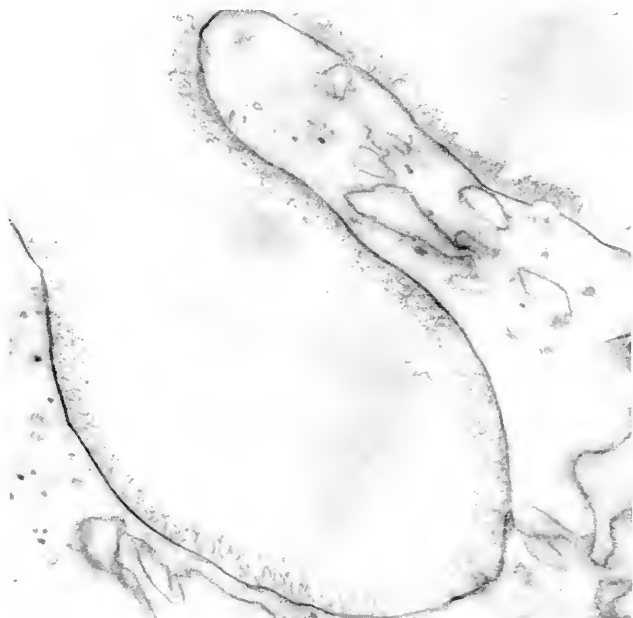


FIG. 3. Binding of ferritin to surface coat at pH 4. The specimen was washed for 2 min. at pH 6.5; most of the ferritin has been removed.

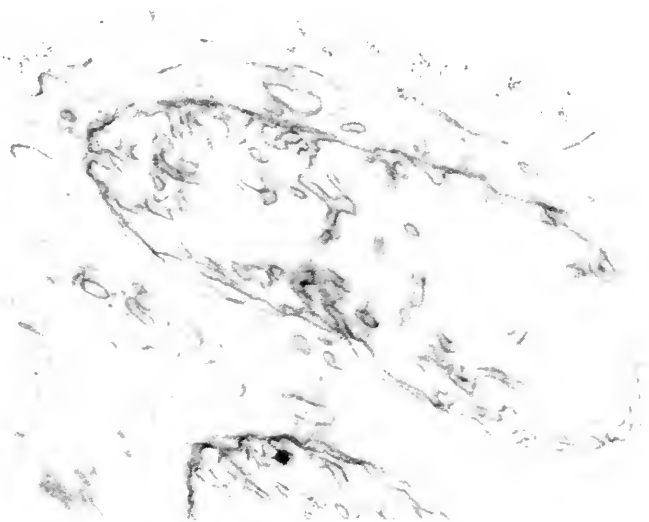


FIG. 4. Binding of methylated ferritin to surface coat at pH 4. The binding persists after washing at pH for 5 to 10 min.

the particles are held in a layer about 1000 Ångströms thick just outside the cell membrane, which is not itself stained by osmium.

Figure 3, again of a specimen treated with ferritin, shows the effect of 2 min. of washing at pH 6.5. Most of the ferritin has disappeared. Longer washing (for 3 to 4 min.) completely removed the protein. In this instance the layer which binds protein is seen as a faintly osmiophilic, fibrillar substance [15]. Usually this substance, the mucous coat, is not well stained by osmium. Its fibrillar character is variable, and may well be an artifact of fixation.

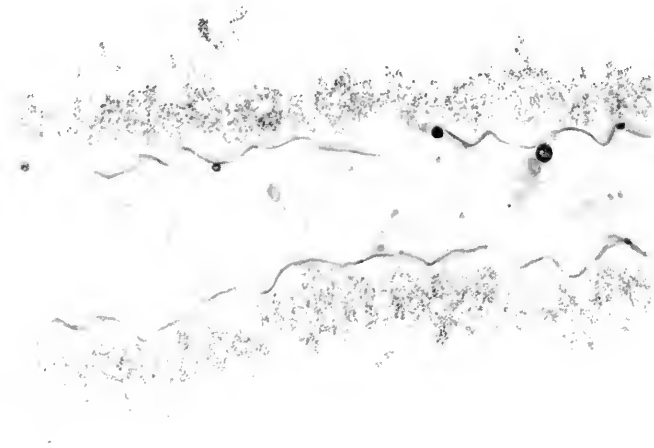


FIG. 5. Binding of methylated ferritin persists after washing at pH 6.4 for 5 to 10 min.

Figure 4 is a micrograph of a specimen treated with methyl ferritin at pH 4, and washed at the same pH. The binding persists, whether the specimen is washed at pH 4 or pH 6.4 (Fig. 5).

These comparative studies demonstrated that binding depends upon the net charge carried by the protein, but gave no direct information about the chemical nature of the mucous coat. By working with mass cultures of *Chaos chaos* in 10 to 50 g. lots, it has been possible to isolate, after tryptic digestion, an acidic, metachromatic polysaccharide. A preliminary analysis suggests that it is a sulphated polyglucose, but this must be confirmed by further investigation.

CHANGES WITHIN VESICLES AFTER UPTAKE OF FERRITIN OR METHYL
FERRITIN

The second question asked at the beginning of this work was: what happens to the pinocytosis vesicle, and to its contents, after it is taken into the cell? Amoebae bearing bound ferritin or methyl ferritin were warmed to room temperature in the wash medium at pH 4. The cells began to change shape, to move, and the orange-stained coat substance was seen to collect in smaller regions of the surface, most commonly in the tail region. Pinocytosis occurred rapidly. The cells were kept at neutral pH,

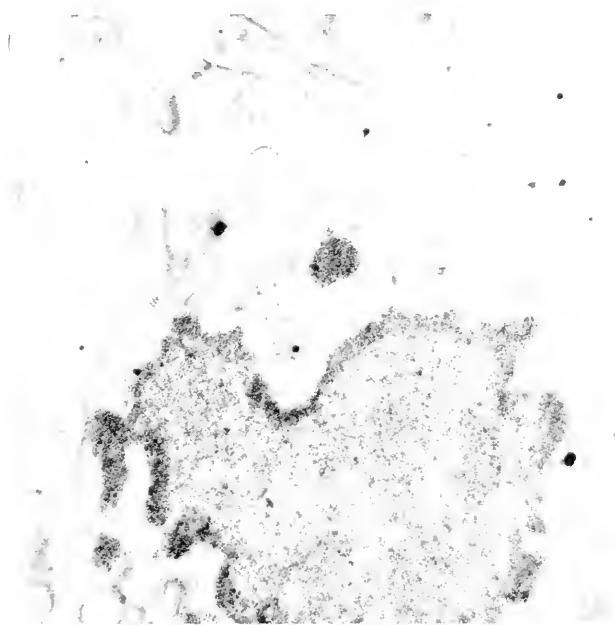


FIG. 6.

and individual specimens were fixed at intervals from 1 hr. to 48 hr. after uptake. When sections were examined by electron microscopy, a complex sequence of morphological changes was found to have occurred. Five features of the process may be described in summary form:

1. Within an hour after a pinocytosis vesicle is formed, ferritin is released from the carrier substance, and collects in irregular masses, usually in the centre of the vesicle. Methyl ferritin, by contrast, remains bound to the carrier substance for the entire 48 hr. period of observation. This difference, because it parallels the effects already described in washing experiments at different pH values, is thought to indicate a rise in pH within the vesicle.

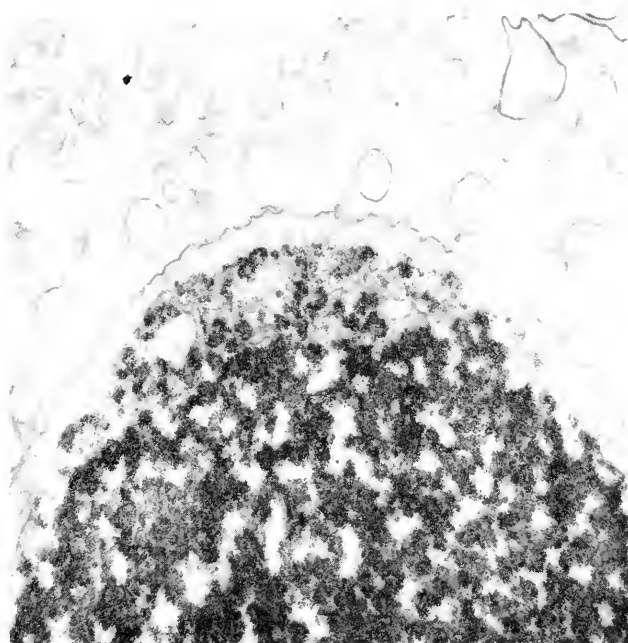


FIG. 7.



FIG. 8.

FIGS. 6, 7 AND 8. Successive stages in the evolution of vesicles containing ferritin, from 1 to 48 hr. after uptake. See text for description of changes which occur.

2. In vesicles containing ferritin, the carrier substance appears to become detached from the membrane proper, and to break up into irregular masses. When the carrier substance is bound to methyl ferritin, however, the entire layer maintains its structural integrity, is detached from the membrane proper, and becomes highly folded and crumpled by 48 hr.

3. The osmiophilic membrane which limits the vesicle remains intact. No pores or holes are seen, but many microvesicles appear attached to the

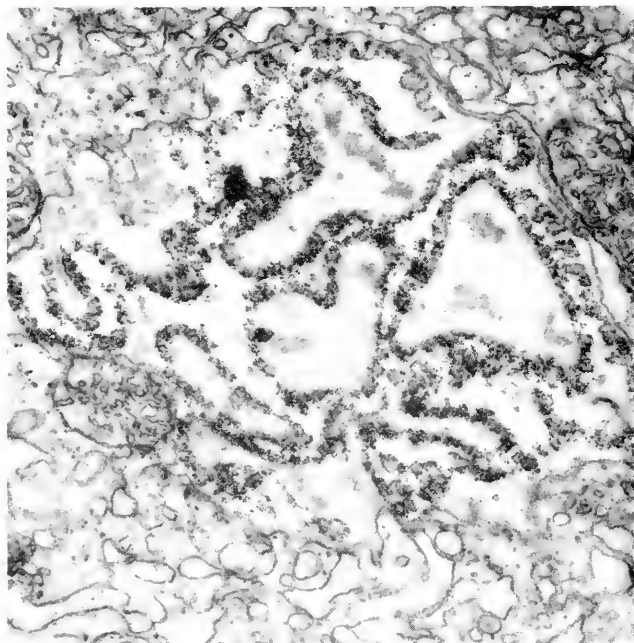


FIG. 9.

outer or cytoplasmic side of the limiting membrane. Also, complex membranous structures, resembling myelin figures, accumulate within the original vesicle.

4. Neither ferritin nor methyl ferritin particles are seen in any part of the cell except the pinocytosis vesicles. Even in amoebae which have carried large numbers of vesicles for 48 hr., the ground cytoplasm, mitochondria, contractile vacuoles and nuclei are consistently free of ferritin or methyl ferritin. The microvesicles, a few hundred Ångström units in diameter, which fill the cytoplasm and are intimately attached to the pinocytosis vesicles, never have been found to contain ferritin or methyl ferritin.

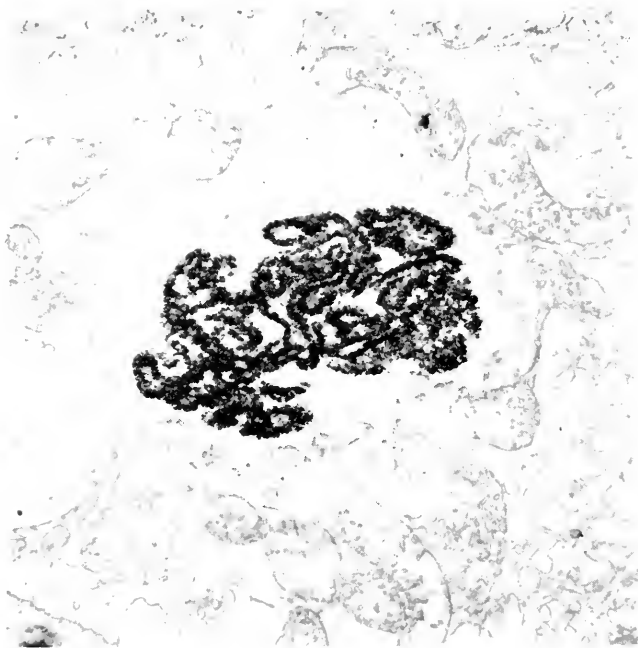


FIG. 10.



FIG. 11.

FIGS. 9, 10 AND 11. Successive stages in the evolution of vesicles containing methylated ferritin, from 1 to 48 hr. after uptake. See text for description of changes.

5. In 48 hr. many of the ferritin particles within the vesicles lose their discrete character and form dense amorphous masses, as though their protein coats were removed.

These features are illustrated in Figs. 6, 7, and 8, which show successive stages encountered in vesicles containing ferritin, and in Figs. 9, 10, and 11, of comparable stages in vesicles containing methyl ferritin.

Conclusion

These studies on ferritin and methylated ferritin demonstrate that the initial reaction of proteins with the cell surface depends upon net charge effects, that the binding step may be temporarily uncoupled from the subsequent stages of pinocytosis, and that neither free ferritin nor bound methyl ferritin escapes in recognizable form from the pinocytosis vesicle. Such observations, when considered with the experimental evidence already available from other studies, lead to the following view of pinocytosis in amoebae:

Pinocytosis is a co-ordinated sequence of three main processes or stages. The initial binding is an ion exchange reaction, which is capable of some selectivity and of concentrating positively charged substances from the environment. The binding reaction, under normal conditions, sets off the active process of vesicle formation. In this stage, both the membrane proper and the coat substance, with some free fluid as well, are carried into the cell as vesicles are pinched off. This process is metabolically linked, as the first is not, and appears to depend on cytoplasmic contractility in a way which as yet has not been studied adequately.

The third stage comprises a complex series of morphological and chemical events within the cell. The lipoprotein membrane, the mucopolysaccharide carrier substance, and the ingested substances are all modified, each in a different way. The evidence, though by no means complete, suggests that the changes include the digestion of protein and the breaking up or partial digestion of the mucopolysaccharide carrier. This implies the accumulation of hydrolytic enzymes within the vesicle, and supports the idea, for which there is as well morphological evidence, that the pinocytosis vesicle is fundamentally the same as the normal food vacuole in which the amoeba digests his prey [16].

From what is known of the fate of smaller molecules, such as [^{14}C]-glucose [17] and ribonuclease [18], it seems that such substances do pass readily from the primary vesicle into the ground cytoplasm, or into the microvesicles which are formed in great numbers from the primary vesicle. Since neither free ferritin nor bound methyl ferritin escapes the vesicle, such exchanges cannot be the result of a gross breakdown of membrane

structure or function. The cell does not at any stage relinquish its control over permeability; there must be, therefore, highly selective exchange mechanisms operating in both directions across the vesicle membrane, or between the primary vesicle and the microvesicles.

If this general view is correct, it follows that active transport mechanisms are essential elements within the overall process of pinocytosis, but the two phenomena are not strictly equivalent. The debate between physiologist and morphologist may be resolved by considering pinocytosis (in this instance, of protein) to include the entire sequence from the initial binding reaction to the point of metabolic utilization. Alternatively, it may be desirable to retain the somewhat arbitrary morphological definition of pinocytosis as extending only to the formation of vesicles, and to speak of digestion and assimilation as subsequent processes. In either case, the mechanisms of trans-membrane exchange, and the role of the microvesicles within the cytoplasm, remain to be investigated.

Acknowledgments

The authors are indebted to Miss Marianne Pieren and to Mrs. Diane Evans for technical assistance.

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Discussion

ALLEN: In the case of tissue cells it is possible to see especially in time lapse movies that the formation of pinocytotic vesicles is dependent upon the formation of pseudopodia, what appears to be a sort of "chewing" movement of the hyaloplasmic ruffles of tissue cells. I wonder if there is the possibility that the same might actually take place in amoeba and have escaped notice. Have you looked into this?

MARSHALL: We have looked into this, but have not seen in amoebae quite the process you describe, nor have others who have studied this more thoroughly, I believe. I would only agree with the point made by Dr. Holter in his review: different cell types show different morphological patterns of uptake. And even in one cell type, the amoeba, different agents invoke different responses (as Chapman-Andresen has shown). I don't know whether we can equate all forms of pinocytosis; differences exist, but these may be less important than the general similarities.

PORTER: What do you regard as the source of the hydrolytic enzymes acting in these vesicles? Is it possible that they are contained in some of the smaller vesicles that you see associated with the surface of the microvesicles?

MARSHALL: It is possible. We don't really know which way the microvesicles are going. All those we see clustered around a big vesicle, in micrographs, contain something which in density and texture closely resembles the substance within the larger vesicle. Also, the microvesicles are found sometimes in a row, like a string of pearls attached at the end to the larger vesicle. From these points, it seems more likely that they are being detached from the larger vesicle, but I agree that this sort of evidence by no means settles the question. We must still say that transport in and out of this chamber may be "transmembrane" transport in the strict sense, or may be achieved by the addition or subtraction of microvesicles.

GOLDACRE: I am interested to see that there is no evidence of very tight packing of your ferritin molecules on the outside of the membrane which might indicate a tendency to expand the outside and thus cause a mechanical invagination of the vesicles. I wonder if you can see in any of your pictures evidence of that or anything else which might suggest a mechanism?

MARSHALL: There is evidence suggesting expansion of the coat substance. Schumaker's kinetic studies, you may recall, showed a brief second stage of protein uptake, as though the first binding led to the appearance of new binding sites. The ferritin work points in the same direction; when an amoeba is treated in the cold and washed, the entire surface is covered by ferritin initially. As the cell warms up it changes shape, the coloured material accumulates in smaller regions, and the greater part of the cell surface becomes clear. A few minutes after clearing, the new surface will again bind ferritin or methylferritin. This implies that new surface material, and probably new membrane as well, is formed very rapidly. The amoeba surface is a dynamic, continually renewed structure, and we think this is particularly so at the tips of advancing pseudopodia. All this can be seen with living cells in the light microscope. At the level of fine structure, it may be explained by a fusion of microvesicles into the original membrane, the contents of the microvesicles becoming new coat substance. We have seen occasionally something to suggest this in electron micrographs, and hope to find out if it is true.

MITCHELL: I should like to congratulate Dr. Marshall on a beautiful piece of work. It makes one feel that the phrase "membrane transport" which we all keep on using has a double meaning. We are speaking on the one hand of "membrane-transport" and on the other hand of "transport membrane". Dr. Marshall has just used the expression "transmembrane transport", and this is very descriptive of what we usually mean by membrane transport (although we should more logically say "transmembrane port"). The other kind of transport in which the membrane itself is transporting and transported we ought, perhaps, to call "cis-membrane transport"!

HOLTER: Just very briefly I would like to answer Dr. Allen's question from before. While we have never seen in amoebae movements comparable with the undulating movement that occurs in tissue culture cells, we have seen something else that might be related to it and that is a peristaltic movement down along the invagination of the amoeba surface. This peristaltic movement has been observed in a time lapse film that Mrs. Chapman-Andresen made in Glasgow some years ago. Unfortunately the film is technically not good enough to be published or shown, but this special feature was rather distinct in several of the sequences.

ALLEN: I would like to introduce one word of caution regarding the question of the lability of the membrane in the amoeba. It may be quite true that under special circumstances, such as during feeding and during pinocytosis, membrane is indeed formed; the fact that the cell can change from almost a sphere into a long cylinder in a matter of a few minutes indicates that the cell can form a new membrane. However, there is a vast amount of evidence in the literature showing that during normal locomotion there is no membrane formed at the front end of an amoeba. This evidence was gathered chiefly by Schaeffer and by Mast. They showed essentially that a particle placed on the surface of an amoeba, let us say a quarter of the way back from the tip, remains in a constant position with regard to the tip as an *A. proteus* type amoeba advances. However, if you watch particles on the tail surface, their behaviour is not quite according to expectation, in terms of the membrane being pulled forward on the surface of the amoeba. By and large, it can be said that during normal locomotion there is no mass formation of membrane.

HOLTER: This fits very well with the fact that in amoebae pinocytosis and locomotion are antagonistic features. A amoeba that crawls will not pinocytose, and vice versa.

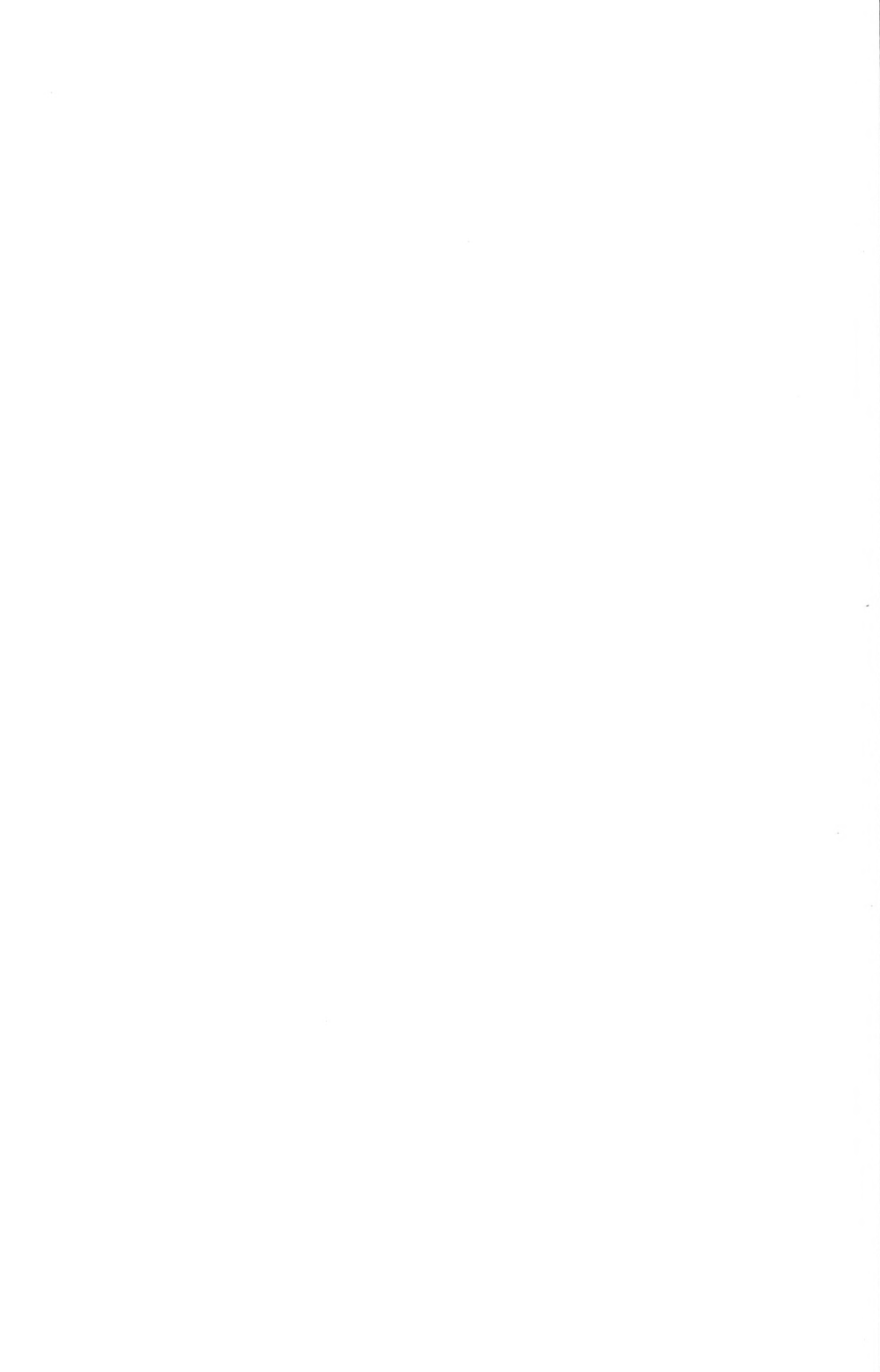
MARSHALL: I think there are many unsettled questions in this. But we are still left with the finding that amoebae can rapidly form new binding material. We don't know what is happening to the membrane proper, but at any rate there is movement and renewal of the surface coat, and in a way it seems simpler to think of membrane and coat as moving together. We have no direct evidence, so I will have to leave it very open as to how this is done by the amoeba.

GOLDACRE: With regard to the point made by Dr. Allen about the forward motion of the membrane as indicated by carbon particles, I think there may be a quite different interpretation of this motion, and that they are not strongly attached. If one attaches a series of *oil drops* to the membrane, which can be seen to be firmly attached because of their contact angle of 90° , they do not move forward. They are overtaken by the tail and are squeezed off eventually at the rear; so that I

think that the movement of carbon and carmine particles apparently on the membrane does not indicate beyond doubt, the movement of the membrane.

ALLEN: If you place a micro-needle through an amoeba, carbon particles attached to the membrane behind the needle will move around the needle. On the other hand, when you put an oil droplet on the surface, it apparently makes a firm contact causing the membrane to adhere to the ectoplasmic tube at this point, in the same way that the membrane adheres to the ectoplasm tube on the bottom of a pseudopod. Thus I still think that the membrane slides forward over newly forming ectoplasmic tubes.

DAVIS: I think it is fair to say that the fact that papers on pinocytosis were placed in different parts of this Symposium is perhaps an indication that we are not yet quite clear on the function of pinocytosis in the cell.



Comparative Study of Membrane Permeability

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The ultimate purpose of studies on the permeability of living membranes is to obtain information concerning the organization and the chemistry of such membranes. It is a well-known fact that cellular membranes actively transport not only inorganic ions but also small organic molecules. In fact the number of functions attributed to cellular membranes is increasing rapidly with the constant progress in that field. One wonders therefore how these various mechanisms are organized at the cell surface.

Thus I should like to discuss what could be called the functional structure of a living membrane, i.e. the way some of the functions so far identified are organized and distributed at the cell boundary. As I will show later, it is possible to identify a living membrane, knowing its permeability properties in exactly the same way as a systematician identifies a species using morphological features. This is the reason why I suggest defining what we could call the permeability characters of a living membrane. As far as inorganic ions are concerned, the active transport of Na, Cl, etc., for instance, are permeability characters. It is the same for the properties of passive permeability to Na, K, Cl and so on.

The comparative study of membrane permeability offers many interesting aspects since it may help us to establish not only the distribution of the permeability characters in the animal kingdom but also their organization within the cell membrane. Last but not least, having established the functional organization of a living membrane one still has to define the chemical nature of the molecular architectures responsible for the various permeability characters.

I should like to illustrate these various points by discussing some of the recent work we have been doing, Miss M. Baillien and I, in Professor Florkin's laboratory.

Let us first examine the results of potential difference measurements performed at various levels of the digestive tract in some animal species.

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The potential difference is measured on isolated segment using the method of Wilson and Wiseman [1] or a new method described elsewhere [2]. In the latter case, the fragment of organ is opened flat and mounted between two chambers. When anatomically possible, the muscle layers are stripped off the epithelium.

It can be seen (Table I) that:

- (a) the lumen of the digestive tract is negative with respect to the serosal fluid,

TABLE I

POTENTIAL DIFFERENCE ACROSS THE EPITHELIUM OF THE DIGESTIVE TRACT IN VARIOUS ANIMAL SPECIES

The potential difference is recorded on isolated preparations: isolated sac (A) or method of Baillien and Schoffeniels [2] (B) with (S) or without stripping of the muscle layers. Both sides of the preparation are bathed with physiological saline. The sign refers to the serosal fluid.

Species	Organ	Potential difference in mV	Methods
Guinea-pig (<i>Caecia porcellus</i> L.)	jejunum	0.5-2	A
	ileum	0.5-2	
	colon	17-20	
Rat (<i>Rattus norvegicus</i> Exl.)	ileum	0.5-2.5	A
	colon	30-40	
Rabbit (<i>Oryctolagus cuniculus</i> L.)	ileum	1.5-2	A
	colon	5-10	
Goldfish (<i>Carassius auratus</i> L.)	small intestine	3.5	B
Carp (<i>Cyprinus carpio nudus</i> L.)	small intestine	3.5	B
Trout (<i>Salmo irrideus</i> Gibbons)	small intestine	0.5	B
Terrestrial turtle (<i>Testudo hermanni</i> G. F. Gmelin)	gastric mucosa	37	BS
	small intestine	2-4	
	caecum	10-15	
	colon	20-50	
Water turtle (<i>Emys orbicularis</i> L.)	small intestine	1.5-2.5	BS
	colon	12-20	
Frog (<i>Rana temporaria</i> L.)	gastric mucosa	30-50	BS
	rectum	10-50	

(b) while the potential difference is around 30 mV across the gastric mucosa, the caecum and the colon, it is only a few mV across the epithelium of the small intestine.

These observations raise two important points: what is the origin of the potential difference recorded and why is the potential difference across the small intestine so low? We have therefore measured the fluxes of Na in the small intestine and in the colon of *Testudo hermanni* G. F. Gmelin, using the double labelling and the short-circuit current techniques [3]. We have chosen the turtle because in this species it is quite easy to strip the muscle layers from the epithelium [2].

TABLE II

INFUX AND OUTFUX OF NA ACROSS THE ISOLATED EPITHELIUM OF THE SMALL INTESTINE AND THE COLON IN THE TURTLE *Testudo hermanni* G. F. GMELIN

The epithelium is bathed with physiological saline on both sides. Results obtained when 2-4,dinitrophenol (DNP) is applied are also given. The fluxes, the net flux and the short-circuit current are expressed in $\text{mcoul. cm}^{-2} \cdot \text{H}^{-1}$. C = control. DNP concentration: 0.1 mM. Experimental periods: 1 hr.

	Influx	Outflux	Current	Net Flux
	small intestine			
C	492	401.4	41.6	90.6
C	540.4	437.1	40.1	103.3
DNP	492	329	32.2	163
	419	314	28.5	105
	colon			
C	174	42.4	104	131.6
C	121.6	34.7	61	86.9
DNP	42.4	28.9	33	13.5
	50.2	50.2	13	0

Table II gives the results obtained. In this Table influx means flux from mucosal to serosal side while outflux means the flux in the opposite direction.

It is clear from the results given that there is an active transport of Na from the mucosal to the serosal side in both small intestine and colon. The values of the short-circuit current are always smaller than the corresponding values of net flux. This means that a cation must be transported from the serosal to the mucosal side or that an anion is transported in the opposite direction. The flux values are higher in the small intestine than in the colon. Since the DNP inhibits the influx in the colon while it inhibits, at least partly, both influx and outflux in the small intestine, this could mean that part of the outflux, in the latter case, is due to active transport.

As shown in Table II the extent of the inhibition is very different depending on whether we consider the colon or the intestine. This could be explained by a very high passive permeability to Na in the intestine.

TABLE III

EFFECT OF NA AND K CONCENTRATIONS ON THE POTENTIAL DIFFERENCE ACROSS THE ISOLATED EPITHELIUM OF THE COLON AND THE SMALL INTESTINE IN THE TURTLE *Testudo hermanni* G. G. GMELIN

M = mucosal side; S = serosal side. Potential difference in mV; the sign refers to the serosal side. Explanations in the text.

Exp.	Small intestine			Colon		
	M	S	PD (mV)	M	S	PD (mV)
A	RCl	RCl	1	RCl	RCl	15
	RSO ₄	RSO ₄	1	RSO ₄	RSO ₄	34
B	RCl	RCl	1	RCl	RCl	20
	RSO ₄	RSO ₄	1	RSO ₄	RSO ₄	45
	RSO ₄ -Na 10	RSO ₄	26	RSO ₄ -Na 10	RSO ₄	25
C	RSO ₄	RSO ₄	1	RSO ₄	RSO ₄	30
	RSO ₄	RSO ₄ -Na 10	30	RSO ₄	RSO ₄ -Na 10	60
D	RCl	RCl	3	RCl	RCl	11
	RCl-Na 10	RCl	18	RCl-Na 10	RCl	5
E	RCl	RCl	0.5	RCl	RCl	38
	RCl	RCl-Na 10	20.5	RCl	RCl-Na 10	40
F	RSO ₄	RSO ₄	1	RSO ₄	RSO ₄	20
	RSO ₄ -K 10	RSO ₄	1	RSO ₄ -K 10	RSO ₄	20
G	RSO ₄	RSO ₄	1	RSO ₄	RSO ₄	22
	RSO ₄	RSO ₄ -K 10	2	RSO ₄	RSO ₄ -K 10	14
H	RSO ₄	RSO ₄	1	RSO ₄	RSO ₄	57
	RSO ₄ -Na 10	RSO ₄	26	RSO ₄ -Na 10	RSO ₄	48
	RSO ₄ -Na 10-K 10	RSO ₄	12	RSO ₄ -Na 10-K 10	RSO ₄	48
I	RSO ₄	RSO ₄	1.5	RSO ₄	RSO ₄	13
	RSO ₄	RSO ₄ -Na 10	20	RSO ₄	RSO ₄ -Na 10	21
	RSO ₄	RSO ₄ -Na 10-K 10	12	RSO ₄	RSO ₄ -Na 10-K 10	19
J	RSO ₄	RSO ₄	0.5	RSO ₄	RSO ₄	35
	RSO ₄	RSO ₄ -K 10	1	RSO ₄ -K 10	RSO ₄	35
K	RSO ₄	RSO ₄	0.5	RSO ₄	RSO ₄	23.5
	RSO ₄ -K 10	RSO ₄	0.5	RSO ₄	RSO ₄ -K 10	22.5
L	RSO ₄	RSO ₄	0.5	RSO ₄	RSO ₄	32
	RSO ₄	RSO ₄ -Na 10	21	RSO ₄	RSO ₄ -Na 10	40
	RSO ₄ -K 10	RSO ₄ -Na 10	24	RSO ₄ -K 10	RSO ₄ -Na 10	40
M	RSO ₄	RSO ₄	0	RSO ₄	RSO ₄	43
	RSO ₄ -Na 10	RSO ₄	32	RSO ₄ -Na 10	RSO ₄	30
	RSO ₄ -Na 10	RSO ₄ -K 10	26	RSO ₄ -Na 10	RSO ₄ -K 10	12.5

Let us consider now the effects of a modification in Na and K concentrations on the potential difference across both intestine and colon (Table III). In these experiments Cl is generally replaced by an equivalent amount of the non-penetrating anion SO₄. When Na or K are removed, they are replaced by an iso-osmotic amount of sucrose. RCl means a physiological

saline having the following composition: 113 mM NaCl; 1.9 mM KCl; 0.45 mM CaCl₂; phosphate buffer pH 7.0. RSO₄ means the same saline in which Cl has been replaced by an equivalent amount of SO₄. RSO₄-Na/10 means sulphate saline containing ten times less Na than the physiological saline. RSO₄-K × 10 means sulphate saline containing ten times more K than the physiological saline. RSO₄-Na 10-K × 10 is a sulphate saline containing ten times less Na and ten times more K than the physiological saline. RSO₄-K 10 is a sulphate saline containing ten times less K than the physiological saline.

It can be seen that:

(1) The replacement of Cl by SO₄ is without any effect on the potential difference across the small intestine, contrary to the situation in the colon where the potential difference increases (expt. A).

(2) In a sulphate saline, a decrease in Na concentration in the mucosal solution results in the inversion of the potential difference across the small intestine. In the colon, we observe a decrease in potential difference (expt. B).

(3) In sulphate saline, a decrease in Na concentration in the serosal solution increases the potential difference across both small intestine and colon (expt. C).

(4) In normal saline, alterations in Na concentrations in the mucosal or serosal solutions, result in the same variations as in SO₄ saline. The variations are nevertheless not so important (expts. D and E).

(5) In sulphate saline, if the concentration in K is increased in the mucosal solution, the potential difference across the colon and the small intestine is unaffected (expt. F).

(6) However, it is possible to show that the K concentration in the mucosal solution affects the potential difference in the intestine if we decrease first the Na concentration. The result is an inversion of the potential difference. If one then increases the K concentration, the potential difference decreases in the small intestine while it stays constant in the colon (expt. H).

(7) In sulphate saline, an increase in K concentration in the serosal solution results in an inversion of the potential difference across the small intestine and in a decrease of the potential difference in the colon (expt. G).

(8) In sulphate saline, a decrease in Na concentration in the serosal solution increases the potential difference across the small intestine and the colon. If the K concentration is increased, the potential difference decreases (expt. I).

(9) In sulphate saline, a decrease in K concentration in the mucosal or in the serosal solution is without appreciable effect on the potential difference across both the colon and the small intestine (expts. J. and K).

(10) In sulphate saline, a decrease in Na concentration in the serosal fluid increases the potential difference in the colon and in the small intestine. An increase in K concentration in the mucosal solution increases the potential difference across the small intestine while it does not modify the potential difference in the colon (expt. L).

(11) In sulphate saline a decrease in Na concentration in the musocal solution produces an inversion of the potential difference in the small intestine, while it decreases the potential difference in the colon. An increase in K concentration decreases then the potential difference in both tissues (expt. M).

The above observations suggest the following conclusions. Since the replacement of Cl by SO_4 in the solutions bathing both sides of the colon produces an increase in potential difference, it is clear that this epithelium is relatively impermeable* to SO_4 . The epithelium of the small intestine is equally permeable (or impermeable) to Cl and SO_4 since the replacement of Cl by SO_4 has no effect on the potential difference. This result could also be explained if one postulates that the epithelium, although impermeable to SO_4 , is permeable to K and Na. This possibility is most likely to be correct since the other results demonstrate that the epithelium is permeable to Na and K. Moreover, using ^{35}S as tracer, we have been able to show that the permeability coefficient for SO_4 in frog skin, turtle colon and intestine are of the same order of magnitude (unpublished results). Nevertheless the fact that the replacement of Cl by SO_4 does not much affect the magnitude of the potential difference when the Na concentration is modified, seems to suggest that the passive permeability of the small intestine to Cl is low.

The small spontaneous potential difference existing across the small intestine is related to the concentrations of Na and K in the mucosal and serosal solutions: it is thus clear that both mucosal and serosal sides of the epithelium are permeable to these ions. On the other hand, in the colon the mucosal side is permeable to Na, but impermeable to K, while the serosal side is permeable to both ions. The conclusions may be summarized in the following scheme (Fig. 1).

This is a schematic representation for the permeability characters of the cells forming the turtle intestine epithelium (Fig. 1, A and B). The permeability characters of the frog skin are also given, for comparison (Fig. 1, C). The outer membrane is in contact with the mucosal or outside solution while the inner membrane is in contact with the serosal or inside solution.

The outward facing membrane is Na and K selective in the small

* Impermeability must be considered in terms of relativity or in statistical terms of probability. Given a highly sensitive method, any substance can be shown to cross a membrane.

intestine and Na selective in the colon and frog skin. The inner membrane is Na and K selective in the colon and small intestine while it is K selective in the frog skin. The active transport mechanism for Na is located at both outer and inner faces in the small intestine while it is located at the inner membrane in the colon and the frog skin.

Koefoed-Johnsen and Ussing [4] have demonstrated that in frog skin, the active transport of Na is in fact a forced exchange for K. This has not yet been demonstrated in the intestinal epithelium. Such a picture describes satisfactorily how the potential difference develops under a wide variety of conditions. Let us first consider the small intestine cell in the presence of a non-penetrating anion (Fig. 1, A). Na diffuses into the

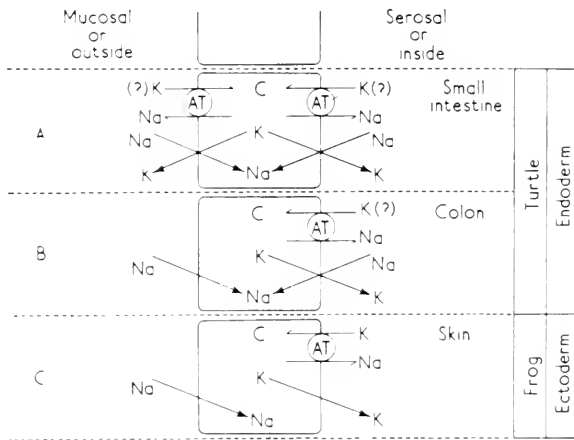


FIG. 1. Schematic representation of the permeability characters of epithelial cells from

- A = turtle small intestine
- B = turtle colon
- R = frog skin

The oblique arrows indicate passive diffusion. AT is the mechanism of active transport with a one-to-one exchange for K and Na in frog skin; this type of exchange has not yet been demonstrated in intestinal cells. The level of the chemical symbols designate the concentration levels of the cations. Explanations in text.

cell from the outside border and gives rise to a diffusion potential which makes the cell positive in relation to the outside. K diffuses out in the opposite direction. The magnitude of the potential difference across the outer border is given by the Goldman equation [21]:

$$E_m - E_c = \frac{RT}{zF} \ln \frac{P_K^m (K)_m + P_{Na}^m (Na)_m}{P_K^m (K)_c + P_{Na}^m (Na)_c} \tag{1}$$

where $Em - Ec$ is the potential difference between the mucosal solution and the intracellular fluid, P is the coefficients of relative permeability for Na and K of the outer (m) membrane. The other symbols have their usual meaning.

It is evident from this equation that P_K^m and P_{Na}^m being equal as well as the ratio of concentration for Na and K, there will be no potential difference across the cell membrane. The potential at the inner border is:

$$Ec - Es = \frac{RT}{zF} \ln \frac{P_K^s (K)_c + P_{Na}^s (Na)_c}{P_K^s (K)_s + P_{Na}^s (Na)_s} \quad (2)$$

The total potential difference across the epithelium is then

$$E = (Em - Ec) + (Ec - Es)$$

E will depend not only on the relative magnitude of the concentration ratios but also on the relative values of the P 's. This is well demonstrated in Table III. Equations (1) and (2) show also why the results of experiments J and K (Table III) are not in contradiction with the conclusion that both mucosal and serosal faces of the small intestine are permeable to K. It is indeed evident that a decrease in the extracellular K concentration will lead to a smaller overall change in potential than an increase.

The situation is more complex if we consider the behaviour of Cl. Since in most cells, the Cl distribution is generally thought to be entirely passive, we would have to assume that in the specific case of the intestinal cell, the cellular Cl concentration is equal to that in the extracellular fluid. But low intracellular Cl concentration could nevertheless be found if the cell possesses a mechanism of active extrusion for this anion located at the inner border. This is certainly the case since we have found (Table II) that the short circuit current is smaller than the net flux of Na. More direct evidence may be found in the results of Durbin *et al.* [5], showing that in the rat small intestine Cl is actively transported from the mucosal to the serosal side (see also [6]). This question will be settled as soon as we have not only measured the flux of Cl with tracers, but also determined with micro-electrodes the exact magnitude of $(Em - Ec)$ and $(Ec - Es)$.

As far as the colon is concerned, the total potential difference may also be related to the sum of two potential differences arising at the outer and inner borders of the cell. Since the spontaneous potential difference increases if SO_4 replaces Cl, we have to introduce, in the Goldman equation [22], the concentration ratio for Cl. Thus

$$E = (Em - Ec) + (Ec - Es)$$

and

$$E = \frac{RT}{zF} \ln \frac{P_{Na}^m (Na)_m + P_{Cl}^m (Cl)_c}{P_{Na}^m (Na)_c + P_{Cl}^m (Cl)_m} + \ln \frac{P_K^s (K)_c + P_{Na}^s (Na)_c + P_{Cl}^s (Cl)_s}{P_K^s (K)_s + P_{Na}^s (Na)_s + P_{Cl}^s (Cl)_c} \quad (3)$$

Equation (3) is in agreement with the results given in Table III.

It is obvious from the results reported here, that the distribution of the permeability characters in the small intestine is different from that in the colon or in the frog skin. The organization and the distribution of the permeability characters is thus an important aspect of cell differentiation. A purely speculative scheme has been proposed to explain the possible evolution of the permeability characters in the epithelial tissues ([7], p. 136). According to this hypothesis the primitive state should be characterized by a mechanism of active transport located at both inner and outer border of the cell. The cell would then evolve toward an asymmetry by losing the active transport mechanism at one of the borders. The results reported here give some experimental support to this conception.

It is also worth noting that the small intestine and colon are differentiated from the same embryonic layer, the endoderm, while the frog skin comes from the ectoderm. Cells originating in two different embryologic layers (colon and frog skin) may thus evolve in the same direction (convergent differentiation).

Without going further into a detailed comparative study of membrane permeability it is apparent that epithelial cells, such as those of frog skin and turtle intestine, possess in principle the same permeability characters as those found in conducting cells, such as electroplax, nerve fibre or muscle, or those found in red blood corpuscles. But one advantage of the epithelial cells studied in the present paper lies in the fact that some of the permeability characters, e.g. passive permeability to Na and K in frog skin and turtle colon, are spatially separated at a microscopic level or are found together in the same membrane (turtle small intestine), while in the nerve fibre for instance, one of them, the Na selective character, appears only for short periods of time (action potential).

Further investigations are now necessary to answer the question whether or not the characters of passive permeability to Na and K are spatially separated in the small intestine cells. One attempt to solve this problem has been made with the isolated electroplax of the electric eel *Electrophorus electricus* L. and the reader is referred to another publication for a more thorough analysis of this matter [7].

Another interesting point raised by the above considerations is the question of the chemical nature of the molecular architectures responsible for the various permeability characters. One may obtain information on this subject by studying the effect of various classes of compounds known to affect the permeability of living membranes. It is a well-known fact that ouabain and some ammonium derivatives affect ionic movement in a variety of cells functionally different or belonging to species situated far apart on the evolutionary scale [7-19]. These results suggest that a common biochemical system could be responsible for all the permeability characters of living membranes. This would thus mean that we could

consider the permeability characters as being heterotypic expression of a common biochemical system, the concept of heterotypy being used here in the sense defined by Mason [20].

An argument in favour of this view is offered by the observation of Tosteson and Hoffman [21] concerning the movement of cations in sheep erythrocytes. It is known that some individual sheep have red cells with high K (HK) and low Na concentrations, while other sheep have red cells with high Na and low K (LK) concentrations. Recent evidence given by Evans (cited in [21]) suggests that the LK character is inherited as a Mendelian dominant. Tosteson and Hoffman found that the active transport of K is four times greater in HK cells than in LK cells, while active transport of Na has not been identified in LK cells. Moreover, LK cells have a greater passive permeability to K and smaller passive permeability to Na than HK cells. Therefore it appears that a single gene controls the permeability characters responsible for both active transport and passive diffusion of K and Na.

Now to conclude. The results reported here show that the various permeability characters are organized or distributed differently in the cells. This distribution is in fact an essential aspect of cell differentiation. Another interesting aspect of the comparative study of membrane permeability is the fact that a common biochemical system seems to be responsible for the permeability characters found in living membranes. Such study may help us to solve the problem of the biochemical mechanism responsible for the cellular differentiation and also to narrow the gaps in our knowledge concerning the structure-function relationships.

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Discussion

DAVIS: I might say that in one aspect of active membrane transport I see no basis for believing that the fundamental mechanism of transport of electrolytes, including mineral ions, is going to be very different from that of stereospecific organic molecules. This is one of the reasons why I am reluctant to use the word "enzyme" in relation to these carriers, because we cannot very well speak of an enzymic conversion of potassium to some product on the way in.



Active Transport and Membrane Expansion-Contraction Cycles

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I. Introduction

That a rhythmically expanding and contracting surface might, by adsorbing and subsequently desorbing various substances, be a mechanism used by the cell to concentrate substances within it was suggested by Goldacre and Lorch [4] and Goldacre [5]. Chemical model experiments showed that substances could indeed be concentrated in this way, and tests with amoebae in various dye solutions, such as neutral red, methylene blue and other basic dyes, indicated that dyes accumulated at that part of the cell where contraction took place, namely the rear end or "tail". This accumulation was shown not to be due to a pH difference between the amoeba's tail and the external medium, for it occurred equally well in media from pH 4 to 8 [6]. Further support was given by Prescott [11], who not only confirmed the original observations but, by preventing plasmagel contraction by converting it all into plasmalol by the application of a hydrostatic pressure of several hundred atmospheres, prevented the accumulation of neutral red by the cell which, however, began to accumulate it when the pressure was released and plasmagel contractions were restored.

The contractile protein hypothesis has been modified and extended by Danielli [3] who suggested that protein chains lying on the outside of the cell membrane with adsorbed substances on them might actually be pulled inside through a micropore by an internal contraction; he further showed that, on the assumption that ATP provided the energy for the contraction (which ATP is known to cause when injected into amoebae [4] and slime moulds [13]) the distribution of phosphatases in various secretory tissues is appropriate to the direction of secretion.

In amoebae, the rhythmic contraction-expansion cycle is obvious microscopically, but other cells, such as red cells, which do not reveal marked rhythmic movements even in time-lapse cinematography, have active transport mechanisms. It is interesting in this connection that a

"shimmering" or vibratory activity of the red cell membrane was reported by Pulvertaft [12], using a special optical technique, which may represent minute contractions of various small regions of the cell membrane. This "shimmering" could be abolished by 0.1 M fluoride, which also abolished the uptake of potassium ions, indicating a possible connection between the two [1]. Recently, also, ATP has been implicated in the maintenance of the biconcave shape of the red cell [10].

In plant cells, a contraction occurring in the membrane at one end of the vacuole in root hairs appeared to be associated with the release of neutral red into the vacuole at that place [5], when the cells were immersed in the dye solution. That the vacuole is implicated in the accumulatory mechanism is indicated by the observations of Brown [2] who showed that active uptake of potassium occurred in the roots of higher plants only at and above the zone of elongation, where vacuoles were present.

In this communication I should like to describe an expansion-contraction cycle in the vacuolar membrane of giant *Nitella* cells, which appears to be associated with an accumulatory mechanism.

In order for an accumulatory mechanism to be effective, there should ideally be two processes: (a) concentration of material, and (b) a valve action or one-way movement, so that accumulated material does not leak away. However, (b) is not absolutely necessary, if the rate of accumulation is high, and the rate of leakage lower; some accumulation would still occur and this appears to be the situation in the accumulation by amoebae of neutral red, which can in time be washed out of the cells by water. *Nitella* cells appear to provide both these mechanisms.

2. Internodal cells

The large chloroplast-lined internodal cells of *Nitella* are not particularly active in accumulating neutral red, accumulating about as much in a few hours as the more active rhizoids accumulate in a few minutes from the same solution. A casual inspection of the cyclosis reveals no obvious regions of cytoplasmic contraction, the cytoplasm circulating at a uniform speed of about 100 microns per second in a slow spiral path up one side of the cell and down the other. In some of the cells, however, interesting phenomena occur at the ends where the cells are attached to their neighbours. Usually, groups of small cells cover and obscure the junction and it is necessary to search through many specimens until a cell favourably placed for observation can be found (Fig. 1, B).

In some of these, it can be observed that the layer of cytoplasm at the end is thicker (d_3 , Fig. 1) than that running into it (d_1), and this thicker layer moves more slowly. This means that the surface area of the vacuolar membrane is contracting at this point. For example, consider unit volume

of cytoplasm of thickness d_1 . This has an area of $1/d_1$. Similarly, when the thickness increases to d_3 , the area decreases to $1/d_3$, and the area contracts to d_1/d_3 of its original area. Measurements of the ratio d_1/d_3 varied from $1/3$ to $1/10$ in different cells. The thickness d_2 of cytoplasm flowing away from the junction was also usually greater than that flowing in, and its speed consequently less. Measurements of the ratio of the speeds were approximately equal to the measured ratio of the thicknesses, and changed from time to time in a given cell, and varied from cell to cell.

The cause of this contraction seems to be as follows: the cytoplasm moves fastest near the cell membrane and slowest near the vacuolar membrane as particles on the outside of the stream can be seen continually overtaking particles on the inside (see also [8]). A tangential force appears to be acting on the cytoplasm in contact with the membrane. This force also appears to be partly associated with the chloroplasts in this kind of cell, for (a) if some of the chloroplasts are removed from their rows by micromanipulation, streaming stops at the gap, and (b) detached chloroplasts can often be seen spinning in the cytoplasm at about 2–3 revolutions per second—even in stationary cytoplasm squeezed out of the cell under oil—indicating a tangential force between chloroplast and cytoplasm (see also [9]).

Chloroplasts are absent from the wall joining two cells, so that the cytoplasm would tend to accumulate here, being driven into an inactive region.

Chloroplasts, however, cannot be the motive force, or the sole motive force, since the rhizoids have no chloroplasts and the cytoplasmic movement seems very similar, though the thickness of the moving cytoplasmic layer is in the region of 1–2 microns compared with 10–70 microns in the internodal cell.

3. Rhizoids

Several active regions in the rhizoid cells where membrane contraction occurred were found, and these regions were, in addition to increase in thickness, characterized by conspicuous wrinkling and vacuole formation.

(A) RHIZOID TIP (FIG. 1, A)

The thickness of the cytoplasm at the tip of the rhizoid cells is about 100 times greater than the thickness of the rapidly moving layer on the rest of the cell. As the rapid thin stream feeds into this deep layer, it slows greatly and masses of wrinkles appear at the point of entry.

(B) JUNCTION BETWEEN RHIZOID CELLS (FIG. 1, C)

The peculiar discontinuity in the shape of rhizoid cells where they join one another (rather like a hand-clasp) gives rise to a thick pool of more slowly moving, wrinkling, cytoplasm.

(C) INERT BAND IN MID-REGION OF RHIZOID CELL (FIG. 1, D)

More seldom, a sudden increase in cytoplasm thickness, accompanied by wrinkling of tonoplast and vacuole formation, can be observed in the mid-region of rhizoid cells. Close inspection indicates that the tangential force on the cytoplasm here ceases to operate—cytoplasmic particles

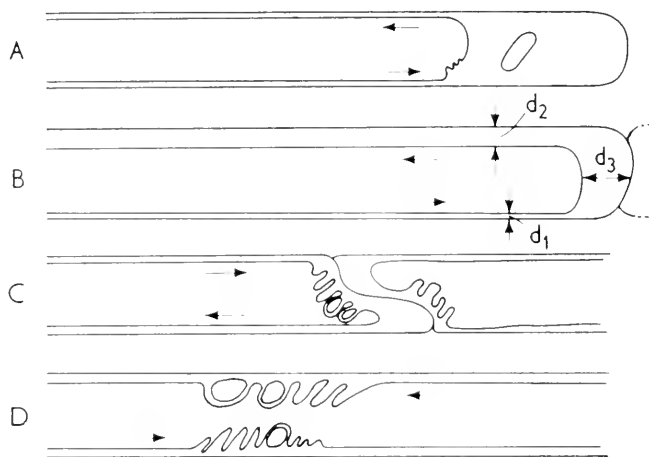


FIG. 1. Diagram showing regions of tonoplast contraction in *Nitella*. A, tip of rhizoid cell; B, elongated internodal cell, at junction with neighbouring cells (chloroplasts are omitted for clarity); C, junction of two cells in rhizoid; D, active patch in mid-region of rhizoid. Note formation of small vacuoles within some wrinkles (C, D). These subsequently unite with the central vacuole.

which elsewhere move fastest near the cell membrane here move slowest near the cell membrane, so that the cytoplasm rides up over itself and forms wrinkles.

4. Discussion

Close inspection of the wrinkles indicated that their area decreased rapidly with time, as if dissolving in the cytoplasm, in a manner similar to the wrinkles in the contracting tail region of the amoeba [4, 5, 7].

The disappearance of surface area of the vacuolar membrane at these regions of increased thickness of the cytoplasm would be accompanied by the simultaneous release there of anything adsorbed on the membrane.

The small vacuoles which usually accompanied the wrinkles and which suddenly formed as the depth of cytoplasm increased could be observed growing in size, then moving to coalesce with the large vacuole of the cell. In the presence of neutral red, concentrated dye could be observed in these small vacuoles.

These small vacuoles are probably induced to form in the cytoplasm by the sudden release into it of a high concentration of accumulated material: it was observed, during work on amoebae, that neutral red injected into the cytoplasm with a micropipette soon induced vacuoles containing the neutral red to form in the cytoplasm; similarly, the dye accumulating naturally in the amoeba, when present in the external medium, at first formed a diffuse cloud in the tail and shortly after became segregated into vacuoles [4, 5]. Vacuoles appear to form, by some unknown mechanism, as a result of high concentrations of various substances.

This coalescence of the induced vacuoles with the large vacuole occupying most of the cell would provide a valve action or one-way effect for the accumulated dye, for the reverse phenomenon—the pinching off of small pieces of the central vacuole—was not observed; so that dye released locally in high concentration by a contraction of the vacuole membrane, on which it was adsorbed, would be captured in these small induced vacuoles, and, by their coalescence with the large vacuole, be prevented from returning to the external medium.

It might be wondered whether the amount of material capable of being adsorbed on the vacuolar membrane could make a significant contribution to the cell's content when desorbed in a contraction. Suppose a monolayer of adsorbed substance weighed one-tenth milligram per square metre (this is about one-tenth of the weight of a protein monolayer). Then in a stream of cytoplasm travelling at 100 microns per second and 30 microns wide, 3000 square microns will disappear per second, releasing 3×10^{-10} mg. to the vacuole per second, and increasing the sap concentration by 0.3 p.p.m. per second, or 2% per day. Hence, if any substances, adsorbable on the vacuolar membrane, succeeded in diffusing passively into the cell in low concentration, they would be concentrated by the local contractions and ultimately captured by the central vacuole by the coalescence with it of the small vacuoles they induced.

5. Summary

In *Nitella* cells exhibiting cyclosis, small localized regions exist where a continuous contraction of area of the vacuolar membrane occurs. These are regions where a sudden fall in speed of streaming occurs, owing to an increase in depth of the stream produced by some discontinuity in the cells, resulting in accumulation of membrane, with wrinkling and its ultimate dissolving into the cytoplasm. Any adsorbed substances would be shed there. A valve action appears to be provided by the formation of small vacuoles in the cytoplasm, apparently induced by the high concentration of substances shed, and containing the substances in high concentration; these small vacuoles coalesce with the large vacuole in the cell.

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Discussion

SCHOFFENIELS: As an extension of what you have said in your introduction about some models of active transport presented by Danielli, I would like to consider the following mechanical model we have been working with lately. It is made of two chambers separated by a hole shaped like a funnel. Now if we place small steel balls in both chambers and if we shake the whole apparatus we get an accumulation of balls in the chamber facing the large aperture of the funnel. This could well be, among many others proposed, a model for a cellular mechanism of active transport. This would imply the existence of a contractile structure in the cellular membrane as well as the presence of a funnel-like pore exhibiting specificity toward an ion species.

ALLEN: I don't think there is any doubt that Dr. Goldacre's observations on dye accumulation in the amoeba are correct. It was described also independently by Dr. Okada in Japan, 1930, but dye accumulation is subject to alternative interpretations. In fact, one might say that the dye accumulation phenomenon is compatible with at least three hypotheses. First, as Dr. Goldacre pointed out in his paper in 1950, the entire surface of the amoeba (i.e. the plasmalemma and the outer layer of ectoplasm) adsorbs quite a bit of dye; by the time the amoeba has moved one cell length, this adsorbed dye has accumulated in the tail. Now if you

follow cytoplasmic inclusions in the outer region of the ectoplasm as markers, you find that they also accumulate over a two-minute period in the same tail region in which the dye has been found to accumulate. Therefore, one expects that the normal pattern in which cytoplasm circulates in the amoeba will cause dye adsorbed to any part of the surface underneath the membrane to accumulate in the tail. There is a second good reason why this accumulation should occur; if you look carefully at the tail of an amoeba you find it contains a great deal of the surface area. The membrane looks superficially like a bunch of grapes, or the surface of large lobose villi. The greater surface area in the tail would lead one to expect greater penetration of dye. A third factor which should be taken into account is that when the dye penetrates, it enters small vacuoles which look very similar to injury vacuoles. These form not only in amoeba but in other cells as well as a result of neutral red treatment. Nowland in 1957 showed not only that neutral red accumulates in vacuoles, but that the cytoplasm in the region of large accumulations of neutral red vacuoles is in an injured state. Goldacre has once reported finding an accumulation of stained particles which so altered the consistency of the cytoplasm that they could be felt as a lump with a micromanipulator needle. If the dye is sealed in vacuoles, it seems unlikely that it would tell very much about molecular events in the ground cytoplasm. In view of these difficulties and alternative interpretations of the dye accumulation phenomenon I believe that one really can't draw any conclusions from it. I would also like to point out again that Dr. Goldacre's views on membrane formation and destruction just do not conform with the results of many published experiments.

GOLDACRE: I should like first to comment on the wrinkles in the amoeba's tail: the area of membrane per unit mass of cytoplasm there would be several times that in most other parts of the cell, but the amount of neutral red which accumulates in the tail may be a hundred or a thousand times as much as you get elsewhere, increasing as time goes on; so I don't think that passive diffusion through the extra area of the wrinkles could account for the dye accumulation in the tail, by several powers of ten. If it were a passive diffusion, as Dr. Allen suggests, the amoeba would tend to become more uniformly coloured with time, say, after the first few minutes; instead, the opposite occurs—the concentration in the tail continues to mount, and the cytoplasm in the rest of the amoeba remains relatively free of dye.

With regard to Dr. Allen's statement that all the ectoplasmic particles accumulate in the tail region: that is not in accordance with my experience nor with the published accounts of Mast and other careful observers; the particles circulate indefinitely around the cell and do not accumulate anywhere.

I don't think I can agree with Dr. Allen's third point because there are many experiments which show that the membrane does not move forwards, including those with oil drops which are attached to the membrane and when the contraction of the tail advances up to them they are squeezed off into the external medium; carmine particles have indeed been shown to move forwards and I have seen that myself, but I don't think they are properly attached to the membrane; you can even see them moving forwards in the external medium separated by 10 or 20 microns from the surface of the cell, but with oil drops there is no doubt that the attachment is firm, for the angle of contact is 90° . I think the movement of carmine

is probably an electrical phenomenon of some kind. If you reverse the electric charge by pre-treating them with polyethylene imine solution, they move in the opposite direction and form a clump on the amoeba's tail.

Another experiment which shows that the membrane does not move forwards is as follows: if you put an amoeba into a narrow capillary tube so that it is squeezed on all sides, becoming thereby about twice its natural length, it continues to crawl through the tube. If the membrane was moving forwards this would not happen. Also, if you put parallel glass fibres on the surface of the amoeba with a micro-manipulator, so that one end rests on the amoeba and the other on the slide (see Fig. A1), then as the amoeba moves forwards those in the rear are pulled together whereas those in the front remain unmoved. Contraction therefore appears to occur in the tail region, with the membrane remaining stationary in the middle portion of the amoeba, and forming *de novo* in the front.

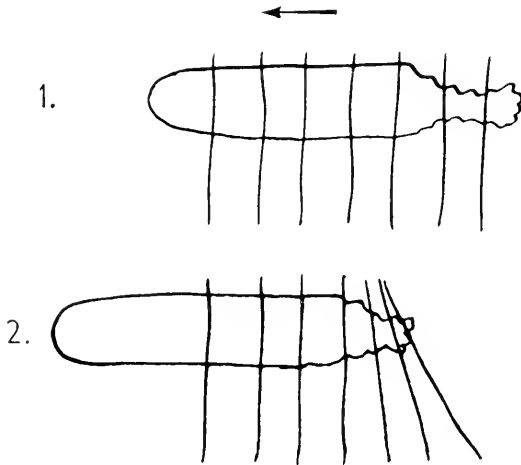


FIG. A1.

ALLEN: In the next issue of *Experimental Cell Research* Dr. J. L. Griffin and I are publishing pictures of a monopodial amoeba which is turning to the left. A carbon particle attached to the left side advances toward the front of the cell, over the hyaline cap and comes to lie on the right side of the pseudopod. I think this experiment very nicely invalidates the idea that a bioelectric phenomenon might be the cause of forward movement of particles attached to the plasmalemma. While an amoeba can form a new membrane (e.g. to replace that lost during phagocytosis), all available evidence suggests that there is not much membrane turnover during active locomotion.

PORTER: I understood that you take the foldings in the tonoplast membrane or the vacuolar membrane to be indicative of contraction, is that so? Could those not also represent a production of excessive membrane at 8 sites on the surface of the tonoplast?

GOLDACRE: Perhaps, but since the motion is towards the site of the deeper region of cytoplasm you would expect that the surface membrane would inevitably

accumulate there and fold or wrinkle because of the reduced surface area available. If you have a stream of water running into a lake, the natural surface film carried down by the stream collapses at the point of entry and you can see this collapse in myriads of parallel striations usually over several metres near the point of entry. In a stream of cytoplasm or water running into a deeper region, unit volume will have an area, if the depth is D_1 in the shallow part, of $1/D_1$, and if the depth is D_2 in the deep part, of $1/D_2$, so the ratios of the surface areas will be D_2/D_1 ; the ratio of these thicknesses may be 10 to 1 or a 100 to 1 in the cell, which means that when cytoplasm with its associated membrane reaches the end of the cell at these turning points, 90 to 99%, of the area disappears giving almost complete desorption of anything that would be adsorbed on it.

ALLEN: I am very curious to ask Dr. Goldacre exactly how he changed the charge of the particles in the experiments he just described a moment or two ago.

GOLDACRE: Carmine particles were suspended in a solution of polyethylene imine hydrochloride (a cationic polymer, which is adsorbed by the carmine) and then washed, and it was shown that the charge had been reversed with wires and a battery; the particles moved in the opposite direction.

DAVIS: It seems to me that the possible mechanism of active transport involving a contractile element does not critically depend on the kind of contractions that are grossly visible. Are these not more of a model system for the micro-contractions which would change the affinity of the substance for the carrier?

GOLDACRE: Yes, there might well be submicroscopical or near submicroscopical contractions in cells such as the red cell; in the red cell I imagine you might possibly have something like a protein chain or fibre perhaps running along the surface and absorbing things and then folding up in the middle of the cell somewhere. There is not much evidence of that, but the shimmering movements associated with active transport, which can be inhibited by various chemicals (described by Pulvertaft and Maizels) indicate that something must be going on there.

MITCHELL: I was worried a bit by Dr. Schoffeniels' model. Just as we can be too easily convinced by what we see as cytologists when we look down the microscope and are confronted by a very attractive picture; in the same way, we must be very careful indeed in drawing conclusions about an attractive everyday macroscopic model and imagining that what happens in the model can happen at the molecular level. The model described by Dr. Schoffeniels, as it is stated without all sorts of additional specifications, is really a Maxwell demon and can't work at the molecular level. And perhaps it would be appropriate to say that this is true of most of the contractile mechanisms of transport that have been proposed. I am not sure that all of those proposed so far are Maxwell demons, but certainly nearly all.

You could legitimately say that the contraction itself can increase the rate at which the transport takes place: but it simply cannot be responsible for the change of free-energy of the molecule which is eventually regenerated after carriage of the molecule through the membrane as a result of its formation of a compound or complex. It simply cannot be responsible for that change.

GOLDACRE: Schoffeniel's model would require a membrane with a valve-like action on the molecular scale. Such asymmetry does not appear to exist in non-

living membranes, though living membranes, using metabolic energy, usually have it in their active transport mechanisms; passive diffusion remains symmetrical, otherwise thermodynamic laws would be violated, as Mitchell indicates. On the other hand expansion-contraction cycles can in fact concentrate adsorbable substances (i.e. change their free-energy state). This happens in the working model I described [*Internat. Rev. Cytology*, **1**, 135 (1952)]—"the inversion tube"—which by adsorbing substances on a large surface and subsequently desorbing them by contracting the surface mechanically, in a small volume, can give you a very large increase in concentration. The energy for the concentration comes from the unequal energies of expansion and contraction of the surface—in contraction, there are adsorbed molecules to squeeze off the surface, and work must be done against the energy of adsorption. In the mechanical model, the energy provided is mechanical; in the living cell, the energy source of the contraction-expansion cycle would be metabolic (probably ATP) as in muscle.

DAVIS: May I direct a comment to Dr. Mitchell? Let us consider a model in which a site with a certain affinity for the permeant is also attached to something contractile; the contraction would distort the site and thereby decrease its affinity. You are not getting work for nothing, as ATP energy would be expended in the contraction. Are you sure that Maxwell's demon is invoked in this kind of model?

MITCHELL: This is evidently a difficult matter to discuss, for it has been chewed over many times and there is not yet general agreement about the conclusions—especially, I believe, since the concepts that we must depend upon are still in process of formulation. I think I would look at it like this. When you postulate a macroscopic model of the propulsion process in membrane transport by considering something like a piece of elastic and how you can manipulate it as a catapult, you are likely to run straight into a conceptual difficulty. Of course, when you let go a piece of elastic it goes flip straight away and one does not think of the thermal activation of this contraction process. If a protein molecule becomes "stretched" or unrolled, the regaining of the configuration that it originally had is, in fact, a diffusion process which must occur by the making and breaking of residual valencies. Moreover, the "stretching" during the phase of the process when it actually occurs must also be a diffusion (or down hill) process, since it would not otherwise happen. In this sense a "contraction" and "expansion" process can be associated with the movement of an ion or other particle through a membrane; but if we are going to "take" the ion and "put it on" to such a system we have to change the ion to "put it on". It has to be attached, not by hand (as a stone may be put in a catapult), but by a chemical bond. When the elastic has got through the membrane (in the ion or molecule type of transport that we are considering here, but not, of course, in group transport) the carried particle has to be detached again—the bond must be broken. The actual work done on the particle in the transport is determined by the difference in free energy in attaching and detaching, and it has no *necessary* connection with the change of configuration of the elastic which happened in between. You may say, as Dr. Davis has just done, that the change of configuration is related in some way to the affinity between the carrier and the particle, but this does not make the actual change of configuration responsible for the work of transport. Although the difficulty with which we are confronted is undoubtedly partly conceptual, it is not, as has sometimes been

suggested, only a matter of words; for, when you come to analyse the transport process you can often distinguish between the free-energy changes involved in making and breaking the intermediate which is going to travel, and the free-energy changes associated with the change of configuration that must happen in between: for example, in the transport of oxygen by the heart and circulatory system. You have two energy considerations. One is the overall free-energy step, up which (or down which) the carried particle must travel—and this simply represents the free-energy difference between the carried species of particles in the phases on either side of the membrane. The other is the activation energy for diffusion (or for “contraction-expansion”) of the carrier-carried particle complex—and this will determine the rate of the process. The elastic process can represent, as it were, a local heating, due to a locally catalyzed exothermic reaction, and this, as I said before, might facilitate the transport. But the elastic process—being no more than a diffusion process at the molecular level of dimensions—cannot actually drive the transport in the normal energetic sense. My criticism of Dr. Schoffeniels’ model, and my statement that it represents, as it stands, a Maxwell demon is based on the fact that it does not include an adequate description of the vital part which must be played by primary or secondary chemical bonding in any ion selective active transport system: he has substituted demons for bonds.

SCHOFFENIELS: Just a few words about Dr. Mitchell’s comment. I do not believe that the mechanical model I have drawn on the board, or any model presented so far, really tells us what is happening in the cell. As a working hypothesis I prefer the carrier hypothesis, but I want to point out that the mechanical model proposed could work at the cellular level. We have first to postulate that the pore is vibrating by means of some kind of contractile machinery, and this would account for the energy spent by the cell in the process of active transport. Then of course the pore must exhibit specificity towards a certain ionic species. This model could be taken as a working hypothesis for active transport, as well as the carrier hypothesis or any other kind of hypothesis since we really do not know anything about the molecular mechanism underlying active transport. Any model thermodynamically sound is then just as good as any other. We nevertheless must be aware of the fact that it is possible to fit the results obtained experimentally with living cells in a great variety of models. This is especially true with regards to the energy requirements of a given model since it is always possible to make *ad hoc* hypothesis as far as efficiency is concerned.

DAVIS: I am not altogether convinced that something involving a contractile element may not be a satisfactory model, and one could even support the proposition that at small enough dimensions a contractile element that changes the shape of a molecule and a group transfer that changes the surface of that molecule converge and become essentially the same thing. It may well be that our thorough understanding of active transport is going to depend on our study of the physical chemistry of macromolecules in such a subtle way that we can really recognize such a convergent phenomenon if it occurs.



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