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CIBA FOUNDATION SYMPOSIUM

ON THE

REGULATION OF CELL METABOLISM

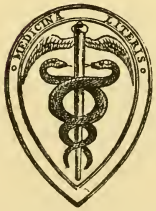
Editors for the Ciba Foundation

G. E. W. WOLSTENHOLME, *O.B.E.*, M.A., M.B., B.Ch.

and

CECILIA M. O'CONNOR, B.Sc

With 109 Illustrations



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THE Ciba Foundation, a unique international institution, owes its inception to the generosity of CIBA Limited, Basle. However, being established under British trust law, it enjoys complete independence in practice and policy.

Under the guidance of its distinguished Trustees, the Foundation offers accommodation to scientists from all over the world at its home in Portland Place. Foremost in its activities is the organization of small conferences, the proceedings of which are published in book form in the manner of the present volume. The Foundation convenes many other informal discussions between research workers of different disciplines and different nationalities and each year invites an outstanding authority to deliver a special lecture. An exchange programme between French and British postgraduates is conducted and a library service is available. Furthermore, the Ciba Foundation attempts in every other way possible to aid scientists, whether they be Nobel Laureates or young graduates making their first original contributions to research.

The purpose of the Ciba Foundation, which is to promote international co-operation in medical and chemical research, is symbolized in the armorial bearings by five interlaced rings representing the continents, a black sacrificial cock (emblem of Aesculapius) holding a medical caduceus, and three regular hexagons for chemistry. Its domicile in London is indicated by the sword of St. Paul and the British lion; the wyvern and the crozier, symbols associated with Basle, refer to the sponsoring firm located in this ancient Swiss town.

PREFACE

Sir Hans Krebs refers in his opening address to the origins of this symposium. It will be clear to readers that in addition to being largely responsible for initiating it, he greatly influenced its organization and, as Chairman, its course. For his encouragement and wise counsel the Director of the Foundation is deeply indebted—an increase in personal and professional indebtedness which goes back to the Director's undergraduate days at Cambridge.

Gratitude is also due in full measure to Professor Dickens, who warmly supported the proposal of the symposium, and gave valued assistance in its preparation.

Although the Editors are accustomed to the task of preparing for publication conference proceedings on very diverse subjects, they are more conscious than ever on this occasion that this book, by experts for experts, may clearly reveal their deficiencies. If the final product is satisfactory, it will be largely due to the ready co-operation of contributors, publishers, and Mr. William Hill, who compiled the index. It is hoped that this work, as Sir Hans has suggested, will prove to be a pioneer book of great value and importance in the coming years.

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List of those participating in or attending the Symposium on "Regulation of Cell Metabolism", 28th–30th July, 1958

W. N. Aldridge	Toxicology Research Unit, M.R.C. Laboratories, Carshalton, Surrey
W. Bartley	Cell Metabolism Research Unit, Dept. of Biochemistry, University of Oxford
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H. Gutfreund	The National Inst. for Research in Dairying, Shinfield
B. Hess	Medizinische Universitäts-Klinik (Ludolf Krehl-Klinik), Heidelberg
Sir Cyril Hinshelwood	Physical Chemistry Laboratory, University of Oxford
F. A. Holton	M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London
H. Holzer	Physiologisch-Chemisches Inst., University of Freiburg im Breisgau
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Sir Hans Krebs	Dept. of Biochemistry, University of Oxford
A. L. Lehninger	Dept. of Physiological Chemistry, Johns Hopkins School of Medicine, Baltimore
F. Lipmann	Rockefeller Inst. for Medical Research, New York

F. Lynen	Max-Planck Inst. für Zellchemie, Munich
B. Magasanik	Dept. of Bacteriology and Immunology, University of Harvard, Boston
C. Martius	Laboratorium für Biochemie, Eidg. Technische Hochschule, Zürich
H. McIlwain	Dept. of Biochemistry, Inst. of Psychiatry, Maudsley Hospital, London
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E. Racker	Division of Nutrition and Physiology, The Public Health Research Inst., New York
P. Siekevitz	Rockefeller Inst. for Medical Research, New York
E. C. Slater	Laboratory of Physiological Chemistry, University of Amsterdam

CHAIRMAN'S INTRODUCTORY ADDRESS

RATE-LIMITING FACTORS IN CELL RESPIRATION

Sir Hans Krebs

Department of Biochemistry, University of Oxford

The origin of this symposium is a conversation between Professor Martius, Professor Lynen and myself which took place in Würzburg, in Professor Martius' laboratory, on July 7th 1955. On July 6th I had given a lecture at Würzburg on the "Steering of Metabolic Processes" (see Krebs, 1956, 1957) and on the day after my lecture Professor Lynen happened to pass through Würzburg and stopped at Professor Martius' Department for a few hours. We talked about the regulation of metabolism and came to the conclusion that it might be useful to have a Ciba Foundation symposium on this subject, and thanks to Dr. Wolstenholme this symposium is now taking place. I am indeed very grateful to him for accepting the proposal and for organizing the conference. I should like to make it clear that he carried the full burden of the preparations. On matters of general policy Professor Dickens gave much helpful advice.

When biologists consider control mechanisms they usually think of hormones and of the nervous system. Certainly these play an important part in some of the mechanisms which control metabolic processes. But control mechanisms also occur in those forms of life which do not possess hormones or nerve cells, such as the unicellular ones. For example, energy may be obtained by fermentation if air is not available, or by oxidation if air is present, the remarkable feature being the controlled stoppage of fermentation by air. Among the most striking regulatory mechanisms of lower organisms are those

which co-ordinate chemical syntheses in relation to growth; they see to it that the quantities synthesized are related to requirements. Such "primitive" control mechanisms of lower organisms are also present in higher animals; they are, in fact, the basic systems upon which the action of hormones or of the nervous system is superimposed. We had in mind that this conference would primarily, though not exclusively, be concerned with these "primitive" control mechanisms. No doubt references will be made to hormones, but any attempt to cover the hormonal field of metabolism extensively would have been beyond the scope of a three-day meeting.

We are fortunate in having at this symposium a number of research workers who are prominent in our field. In view of the wide experience of most of the participants I find it difficult to present a suitable introduction, without being either too elementary, or anticipating future speakers. What I would like to attempt is to describe a few experiments which illustrate the nature of the problem.

This may be generally stated as follows. The chemical systems which exhibit the phenomenon of regulation, i.e. adjustment of activities with reference to a purpose, are all multireaction systems. They consist of a number of relatively simple reactions which can be described by ordinary chemical equations, but these reactions are elaborately interlocked. Interlocking in chemical systems means that individual reactants take part in more than one process, often in many, and to understand the nature of the control mechanism we must unravel the interlocking of the individual reactions. At the present stage one of the main difficulties arises from the fact that the component reactions of the systems which we attempt to analyse are not yet fully known. Moreover, the systems are heterogeneous consisting of many phases or compartments separated by specific barriers and the spatial arrangements of reactants are bound to play an important rôle in the regulation of enzymic processes.

It is probable that regulation of metabolism operates always through variations of reaction velocities: reactions are

accelerated or slowed down rather than brought into play anew. One of the main problems, therefore, in the analysis of regulation is the identification of those component reactions which are rate-limiting.

The system on which I propose to illustrate some aspects of the problem is a heart muscle homogenate. Muscle, striated or cardiac, shows great variations in its rate of respiration depending on the physiological state of the organ. It may increase many times over when the muscle changes from rest to activity. This is not a matter of hormonal action (although hormones contribute to the control of the basal metabolic rate). Variations in the rate of respiration are also encountered in muscle suspensions incubated *in vitro* under different conditions. For example, the rates of oxygen consumption vary with the nature of the available substrate. This is illustrated by Table I, which presents data on the oxygen consumption

Table I

OXYGEN USED BY 4 ML. SHEEP HEART MUSCLE HOMOGENATE CONTAINING 10 PER CENT TISSUE; 20°; 60 MIN.

Substrate added	O_2 *used* (*μmoles*)
none	17·0
pyruvate	26·1
succinate	32·6
L-lactate	21·6
citrate	20·8
α-oxoglutarate	25·4
fumarate	19·1
acetate	21·1
glycogen	15·4

of a suspension of homogenized sheep heart in a saline medium containing potassium chloride, magnesium chloride and phosphate buffer. The tissue concentration was 10% (w/v). Such a suspension absorbs oxygen very rapidly and in order to eliminate diffusion as a limiting factor it is necessary to work at 20°. Even at that temperature 4 ml. suspension may use up to 20 μl. O_2 per min. There is no appreciable increase in the rate of oxygen consumption when glucose or glycogen is

added, but there are considerable increases when acetate, pyruvate or intermediates of the tricarboxylic acid cycle are added.

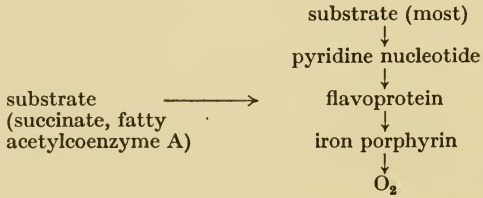
Let us now consider the factors which limit the rate of oxygen consumption in the heart muscle suspension to which no substrate has been added, but which contains much oxidizable material in the form of carbohydrate and lactate. The fact that the oxygen consumption in this system can be increased by the addition of suitable substrates proves that the oxidizing capacity, i.e. the electron transport system from reduced diphosphopyridine nucleotide (DPN) to O_2, is not used to the full, and is thus not a limiting factor in respiration. What, then, is the limiting factor?

The reactions between oxygen and substrates in this system are known to involve iron porphyrins, flavoproteins and pyridine nucleotides. Whilst it is not possible to draw up a comprehensive sequence of reactions, a simplified list indicating the types of reactions which occur can be formulated. These are the following:

substrate + pyridine nucleotide$_{ox.}$	→ substrate$_{ox.}$ + pyridine nucleotide$_{red.}$
DPN$_{ox.}$ + TPN*$_{red.}$	→ DPN$_{red.}$ + TPN$_{ox.}$
DPN$_{red.}$ + flavoprotein$_{ox.}$	→ DPN$_{ox.}$ + flavoprotein$_{red.}$
substrate + flavoprotein$_{ox.}$	→ substrate$_{ox.}$ + flavoprotein$_{red.}$
flavoprotein$^{I}_{red.}$ + flavoprotein$^{II}_{ox.}$	→ flavoprotein$^{I}_{ox.}$ + flavoprotein$^{II}_{red.}$
flavoprotein$_{red.}$ + 2Fe^{3+}-porphyrin	→ flavoprotein$_{ox.}$ + 2Fe^{2+}-porphyrin + 2H^{+}
Fe^{3+}-porphyrin^{I} + Fe^{2+}-porphyrin^{II}	→ Fe^{2+}-porphyrin^{II} + Fe^{3+}-porphyrin^{II}
2Fe^{2+}-porphyrin + 2H^{+} + $\frac{1}{2}O_2$	→ 2Fe^{3+}-porphyrin + H_2O

* Triphosphopyridine nucleotide.

A still further simplified version, indicating the main steps, is the following scheme:



A priori any of the separate stages could be expected to be rate-limiting but under physiological conditions it appears to be generally a reaction involving substrates, and not one of the reactions involving other stages, which is rate-limiting. This applies to animal tissues but not necessarily to micro-organisms. Only under unphysiological conditions, e.g. in the presence of cyanide or other specific inhibitors, does one of the intercarrier reactions become rate-limiting.

As already mentioned, the fact that the oxygen consumption can be increased by certain special substrates which are known to reduce either DPN or flavoprotein shows that the transport of hydrogen from DPN or flavoprotein is not used to full capacity in the absence of these special substrates. It follows that the limiting step is the interaction between substrate and DPN, i.e. the first stage of the electron transport system. When an added substrate increases the rate of respiration it is due to the fact that this substrate reacts more readily with DPN (or flavoprotein, as the case may be) than the endogenous substrate. This, then, is the reason why pyruvate or α-ketoglutarate, or succinate, stimulates the rate of respiration.

The question next arises of what determines the rate of reaction between substrate and pyridine nucleotide (or substrate and flavoprotein). There are two aspects of this question, which I propose to consider. The first concerns the variations of the rate in the presence of one and the same substrate but under varying conditions. Factors which are of importance are obviously the concentrations of the immediate reactants, those of the substrate and of the enzymes and coenzymes attacking it. If, for example, pyruvate were the limiting factor, constant rates of pyruvate removal might be expected when an excess of pyruvate is present and this constant rate would depend on the activity of the saturated enzyme system. A simple experiment, however, shows that other factors can be rate-limiting; the addition of 2 : 4-dinitrophenol (DNP) may cause a doubling of the rate of oxygen consumption and of pyruvate removal (Table II). This proves

Table II

EFFECTS OF 2 : 4-DINITROPHENOL ON OXYGEN CONSUMPTION OF SHEEP HEART MUSCLE SUSPENSIONS. 10 PER CENT TISSUE; 20°; 60 MIN.

	O_2 (μmole) *used by 4 ml. suspension*	
Substrate added	*control*	10^{-5} M DNP
none	17·0	16·1
pyruvate	26·1	51·9
succinate	32·6	46·7
L-lactate	21·6	21·6
citrate	20·8	27·4
α-oxoglutarate	25·4	49·3
fumarate	19·1	17·7
acetate	21·1	31·4
glycogen	15·4	12·6

that the enzyme systems catalysing the interaction between substrate and oxygen are not used to full capacity in the absence of dinitrophenol, in other words that the activity of the pyruvate-oxidizing enzyme system is not rate-limiting.

It is the generally accepted interpretation of the effects of dinitrophenol that this substance uncouples phosphorylation (as Loomis and Lipmann discovered in 1948) and that under physiological conditions the oxidation of reduced pyridine nucleotide is obligatorily coupled with the synthesis of adenosine triphosphate (ATP) from inorganic phosphate and adenosine diphosphate (ADP). This concept offers, in fact, a satisfactory explanation for many observations. Thus, what limits the rate of oxygen consumption and pyruvate removal in the absence of dinitrophenol is not the amount of enzyme, but the level of either ADP or inorganic phosphate. We can exclude the latter in the present experiments because it was added in the medium in relatively high concentrations, and increasing the concentration of phosphate did not stimulate respiration. Under the particular conditions of these experiments the limiting factor must therefore be the amount of ADP available for the coupled oxidation of the electron carriers. The conclusion that dinitrophenol leads to a raised ADP level has, of course, been reached before on the

basis of many other experiments (Lardy and Wellman, 1952; Chance, 1956; Aldridge, 1957).

There are many conditions *in vitro* when dinitrophenol does not stimulate respiration. Tyler (1949) described experiments on rat brain slices showing that the oxidation of glucose, lactate and pyruvate was stimulated by dinitrophenol whilst the oxidation of succinate, citrate or fumarate was not. He also found that the dinitrophenol effect was absent from homogenates though it occurred in slices. The dependence of the dinitrophenol effect on the substrate is also illustrated by data given in Table II. In heart muscle the results obtained differ much from those with brain slices. There is a marked dinitrophenol effect with pyruvate, oxoglutarate and succinate, and a smaller one with citrate and acetate. There is no effect in the suspension to which no substrate has been added but which is rich in carbohydrate and lactate, or after addition of lactate or fumarate.

When dinitrophenol does not accelerate respiration, factors other than ADP and phosphate must be rate-limiting. This is not surprising because two substances (apart from phosphate) are necessary as substrates of oxidative phosphorylation: reduced DPN and ADP. Both these substances behave like catalysts in that they are present in small amounts and require continuous regeneration to remain available. If dinitrophenol acts by speeding up the regeneration of ADP from ATP, it is obvious that this can stimulate respiration only if the speed of this regeneration is not already faster than the speed at which reduced pyridine nucleotide can be regenerated. As soon as the regeneration of reduced DPN is slow its level, rather than that of ADP, may become the limiting factor. Hence, dinitrophenol can be expected to stimulate only when readily oxidizable substrates are available. In the case of other substrates, hydrogen transfer from substrate to carrier, i.e. the activity of the dehydrogenases concerned, must remain rate-limiting even in the presence of dinitrophenol.

A second factor which determines the rate of reaction

between a substrate and DPN is the presence of another substrate. Under physiological conditions it is usual that a variety of different substrates derived from either carbohydrate, or protein or fat, is available. When a mixture of substrates is offered they do not all undergo oxidation at the same time, but one after the other, in such a fashion that the energy supply remains steady, and independent of the quantity of substrates offered. Thus, when a variety of substances are available they compete with each other as fuels. Enzymically this represents in most cases a competition for DPN, and in special cases for other common cofactors. We can test, by direct experiments offering a mixture of substrates, which of the substrates wins in the competition and it is also possible to explain in a general way why they win, namely because of a higher reaction rate under the given conditions. But the physicochemical properties of the enzyme systems which determine the reaction rate have as yet to be analysed.

The competition between substrates for DPN may be visualized as follows. DPN is known to be attached to dehydrogenases as the prosthetic group. If the substrate of the dehydrogenase is available, this prosthetic group will undergo reduction but no further substrate can be attacked until the prosthetic group has been reoxidized by the transfer of electrons to the next stage of the electron transport chain, namely flavoprotein. Thus, if several substrates are present the respective dehydrogenases will be converted to the reduced forms and these are therefore the agents which compete for a common electron carrier.

An example of competition between substrates is the observation that inhibition of dehydrogenases by specific inhibitors does not necessarily change the rate of oxygen consumption of a tissue preparation, because the place of the eliminated substrate can be taken by others. An illustrative case is the effect of parapyruvate on the metabolism of muscle tissue. As Montgomery and Webb (1956) have shown, parapyruvate [$COOH.C(OH)(CH_3).CH_2.CO.COOH$], which

may be looked upon as a structural analogue of α-oxoglutarate, specifically inhibits the removal of α-oxoglutarate. It also inhibits pyruvate removal and oxygen consumption when pyruvate is the only added substrate (Table III), but when

Table III

EFFECT OF PARAPYRUVATE ON OXIDATIVE METABOLISM OF PIGEON BREAST MUSCLE HOMOGENATE

The data refer to 8 ml. suspension; 10 per cent tissue; 20°; 1 hr.

Cup No:	1	2	3	4
Substances added:	pyruvate (0·01 M)	pyruvate (0·01 M)	pyruvate (0·01 M)	pyruvate (0·01 M)
			fumarate (0·01 M)	fumarate (0·01 M)
		para-pyruvate (0·004 M)		para-pyruvate (0·004 M)
Changes (μmoles):				
O_2	−68·8	−16·4	−73·3	−68·3
pyruvate	−21·4	− 2·7	−22·3	−21·0
fumarate + malate	+1·9	+1·3	−15·8	−44·4
α-oxoglutarate	0	0	+2·5	+22·1
citrate	+0·7	0	+1·4	+3·8

both pyruvate and fumarate are added parapyruvate no longer inhibits oxygen consumption and pyruvate removal. It greatly stimulates the removal of fumarate and the data on the changes of metabolites make it clear that those sections of the tricarboxylic acid cycle which operate in the presence of this inhibitor—the stages between fumarate and α-oxoglutarate—proceed more rapidly and thus compensate for the loss of the steps between α-oxoglutarate and fumarate.

In this introductory talk I must limit myself to giving a few examples of the study of the regulation of reaction rates. The fundamental principle involved in these examples is, I think, control by feedback. This differs basically from the mechanisms which operate in hormonal control. Hormones usually (if not always) act through direct effects on an enzyme, either

activating or inhibiting it. In contrast, feedback systems are arrangements in which the rate does not depend on the potential activity of the enzymes, but on environmental factors. The controlled process, as it progresses, creates conditions unfavourable for further progress and thereby causes the rate to slow down. This slowing down, in turn, creates more favourable conditions and thus speeds up the process. This is obvious for the rate control of the total energy supply. Energy expenditure means a conversion of ATP to ADP and phosphate. As each of these products is a reactant in the aerobic and anaerobic energy supply, their formation can accelerate the reactions which lead to their removal. In other words, ADP and phosphate provide the stimulus (or "signal", to use a term of feedback engineering) for the acceleration of the energy-supplying reactions.

Competitive mechanisms in which the pyridine nucleotides and other cofactors are the key substances may also be looked upon as feedback systems. When two substrates compete for one common intermediary catalyst each by its presence creates unfavourable conditions for the reaction of the other substrate. As one of the substrates disappears the second is automatically "fed" to the catalyst so that approximate constancy of catalytic activity is secured. Whether all "primitive" control mechanisms can be classified as feedback systems remains to be seen. This is one of the many questions which I hope this symposium will help to illuminate.

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DISCUSSION

Hinshelwood: An interesting example of the type of change in the limiting process which Sir Hans has been discussing, is found in the oxidation of various carbon substrates by *Aerobacter.* Many of them need to undergo a good deal of adaptation before *Aerobacter* will grow on them as sources of carbon at its optimum rate. During the process of adaptation to a given substrate, or in the comparison of different substrates to which the bacteria are poorly adapted, there is found a good, and more or less linear, correlation between the growth rate and the oxygen consumption (without growth) in the presence of the substrate. However, for the whole group of substrates the same optimum degree of oxygen uptake is reached eventually. It becomes the same and constant, when the cells are fully adapted. Therefore, when they are badly adapted the limiting step is the initial oxidation of the substrate. They reach the constant optimum when the limiting factor has changed, the oxidation rates, which were all different, now being the same for the whole series.

Krebs: Yes, that would be an example of a change of limiting factors under different conditions.

Magasanik: Sir Cyril, does not that actually involve the formation of new enzymes? In other words, adaptation here means the increase in enzyme levels which is then responsible for the greater oxygen uptake.

Hinshelwood: When the requisite dehydrogenase is developed to the maximum extent that the bacteria are capable of, then in all the different cases there is the same constant optimum. This shows that the limiting factor is no longer the rate of substrate oxidation but something else, perhaps the rate of reoxidation of the reduced carriers.

Potter: Sir Hans, in the beginning it sounded as if you made a generalization that most control mechanisms, or most regulations, operated through changes in the reaction rate as if it were only in terms of enzyme activity. To clarify that point, I am sure you would agree that there are many situations in which changes in enzyme amount are very important.

Krebs: I referred to heart muscle or a striated muscle where the amount of enzyme would not be varied when the rate of reaction changes. Of course, there are conditions e.g. in the adapting microorganism, where the amount of enzyme becomes the limiting factor.

Chance: It would be important to define what we mean by velocity of enzyme reactions, and to state how the concentration of enzyme affects the velocity of enzyme reactions. There is a difference between velocities and velocity constants. The velocity constant is the rate of breakdown of the saturated intermediate, but the velocity or the amount of substrate decomposed per unit time will be the product of this velocity constant and the amount of enzyme. The velocity constant with which the intermediate breaks down is a constant characteristic of the enzyme—you cannot say that it is a variable for any particular system.

Potter: Under carefully defined conditions.

Chance: Yes. The product of that velocity constant multiplied by the enzyme concentration is the velocity, which will vary.

Krebs: The velocity constant and the concentration of an enzyme alone do not determine reaction rates. These often depend on the amount of substrate available. For example, in normal animal tissues the activity of most intermediary enzymes is determined by the amount of substrate which arises, because the enzymes have a much greater capacity than is normally required. So you are quite right in drawing attention to the terminology and the need to clarify what is meant when we speak of reaction velocities. The ones that I had in mind are not those depending on certain constants of the enzyme; they refer to the overall reaction which occurs under given conditions.

Chance: It could be called velocity, and it is the consequence of a number of factors. You mentioned the substrate-limited case where the substrate determines the concentration of this intermediate. In other words, the enzyme is not completely saturated with substrate, so that the variable there is the amount of intermediate.

Greville: In most cases where no substrate has been added to tissue slices, DNP has no effect. Even when the endogenous respiration is as high as the respiration in the presence of added substrate, DNP may have no effect. If you add some, but not all, substrates and DNP, then the respiration goes up. I am speaking of tissue slice conditions.

Krebs: You have more experience in this field than I. I have not used slices but, as the examples show, in homogenates also there was no effect unless substrate were added.

Greville: Exceptions were found in the case of slices from liver of fed rats and of a chicken tumour of rather obscure origin.

Chance: Would you say that this applies to excised muscle, i.e. muscle cut away at the tendon and taken from the animal?

Greville: Definitely not. I am speaking entirely of slices.

Chance: It is important to make that distinction. Excised muscle is completely different from tissue slices, and it will show a very large response to DNP.

Greville: Not only that; it also has the peculiarity that anaerobic lactic acid production is vastly increased by addition of DNP.

Krebs: We know that DNP acts in the intact body and causes a raised heat production and oxygen consumption. What you mentioned about slices is a matter of widespread, but perhaps not universal, occurrence.

Siekevitz: There is one example in which the supply of substrates is not limiting, and that is the case of citrate oxidation by mitochondria. You can pile up citrate in the mitochondria (Schneider, W. C., Striebich, M. J., and Hogeboom, G. A. (1956). *J. biol. Chem.*, **222**, 969). There is a very low rate of oxidation which is probably due to the DPNH-linked *iso*citric dehydrogenase (Ernster, L., and Lindberg, O. (1958). *Ann. Rev. Physiol.*, **20**, 13).

Krebs: Citrate is a very special case for two reasons: firstly, because the enzyme limiting its removal acts under physiological conditions

mainly outside the mitochondria and, secondly, there is the complication that any citrate which accumulates upsets the balance of free magnesium ions. As you know, it has been suggested by Raaflaub (1956, *Helv. physiol. pharmacol. Acta*, **14**, 304) that among the rate-controlling factors are magnesium ions, which are needed for a variety of enzymes, and that phosphate, ADP and ATP all react through their magnesium binding. The complexing capacity of ATP is very much greater—by a factor of 50—than the complexing capacity of ADP. Citrate has also a complexing capacity. This has been put forward as a possibility. I have done a few experiments on this, and they do not support it very well. But it has to be borne in mind that it is not easy to make sure that you get your chelating agent into the mitochondrion.

Dickens: In the case of the mitochondrial citrate oxidation, a very interesting survey has been made recently by Ernster and Navazio where it was found that the DPN-specific *iso*citric dehydrogenase in rat liver is localized exclusively in the mitochondria, although there is some TPN-linked dehydrogenase there too (Ernster, L., and Navazio, F. (1957). *Biochim. biophys. Acta*, **26**, 408). The TPN one is, in fact, more active in mitochondrial reduction of coenzyme. But owing to the extremely inefficient transport systems for reoxidizing TPNH the DPN one wins in the overall oxidation of *iso*citrate. According to their figures, which looked rather convincing, the greater part, about 75 per cent, of the oxidation of *iso*citrate in mitochondria of liver should go through DPN and only 11 per cent via transhydrogenase and 7 per cent via TPN. This case is an interesting example where you not only have the effect of substrate concentration, but the choice of which enzyme-coenzyme pathway the oxidation will take.

de Duve: To return to Prof. Potter's question, I think Sir Hans was referring to control mechanisms occurring in systems where the enzyme concentration is constant during the experiment. Prof. Potter was referring to a fairly slow process of adaptation. We have to keep in mind another possibility e.g. where an enzyme is very rapidly converted into an inactive form and can be very rapidly reconverted into an active form, as is known to be the case for phosphorylase. This may occur with other enzymes.

Krebs: This would be the type of case where the hormonal influence is of importance. The activity of phosphorylase depends on the presence of adrenaline.

de Duve: Yes, but several other factors besides adrenaline are known to influence the phosphorylase system. The work on muscle, for instance, indicates that the activity of phosphorylase is also under intracellular control and varies with the state of the tissue.

Slater: With reference to Prof. Dickens' remark on the mechanism of oxidation of *iso*citrate, Dr. Purvis in our laboratory has recently investigated this question with both liver and heart mitochondria (Purvis, J. L. (1958). *Biochim. biophys. Acta*, **30**, 440). His conclusion does not agree with that of Dr. Ernster, but supports the view of Kaplan, namely that the oxidation of *iso*citrate does go through the TPN and the pyridine nucleotide transhydrogenase. If the mitochondria are

really depleted of pyridine nucleotide—and this must be controlled by direct analysis—then *iso*citrate is only oxidized on the addition of both DPN and TPN.

Dickens: That is most interesting. According to Ernster's figures the pyridine nucleotide transhydrogenase could undertake a maximum of about 11 per cent of the total oxidation. I do not understand how the soluble *iso*citric dehydrogenase which, I believe, accounts for 90 per cent of the total activity comes into the story. Is it that the *iso*citric oxidation can take place in the soluble fraction and then the products pass back into the mitochondria?

Siekevitz: It has been observed (Reinafarje, B., and Potter, V. R. (1957). *Cancer Res.*, **17**, 1112) that the TPNH-cytochrome *c* reductase is solubilized very easily from mitochondria, and it is possible that the soluble *iso*citric dehydrogenase is really a mitochondrial enzyme; it may also be very easily solubilized, i.e. during the preparation of the mitochondria there is a loss of this enzyme.

Dickens: In fact you suggest that it is an artifact.

Pardee: As regards the quantities of enzymes in systems of the type you are discussing, there still remains a problem of metabolic control. Presumably these enzymes are in surplus over the amounts required for the reactions to be carried out at physiological rates. One may ask what limits the amounts of enzymes in animal systems. How much enzyme is made, and why is a certain amount made? If there were too few enzyme molecules for any step they would be rate-limiting. The fact that the amounts of many enzymes do not seem to be rate-limiting in animal tissues presumably means that there is something that makes them not rate-limiting, i.e. in excess.

Krebs: Many enzymes are present in quantities in excess of ordinary requirements. There must be some enzymes that are, in fact, rate-limiting.

Pardee: My question is not how greatly in excess they are, but is there a mechanism that provides for the enzymes in only a small excess, or which does limit the amount; i.e. a mechanism which limits the formation of these enzymes? In animal tissues such mechanisms may not be easy to observe because of the animal's homeostatic system.

Dickens: That is certainly a very complicated situation. Perhaps in major pathways there may be no circumstances in which the amounts of enzyme are limiting; but when it comes to minor ones, e.g. the pentose phosphate pathway, they may very well be limiting factors, and the amount of enzyme is probably quite a considerable factor in some circumstances in deciding, e.g., the extent to which the pentose or hexose monophosphate oxidative pathway is, in fact, occurring. This is a typical branching of pathways, in which one regulatory factor must be the relative amounts of the enzymes, as well as the coenzymes, of the hexose monophosphate and glycolytic systems.

Krebs: It is in stages where pathways are initiated that the enzyme amount must be the important part. In general, intermediate products never accumulate. That must mean that there is enough enzyme to cope with the intermediates as they are formed. We have long been

accustomed to the idea that the initiating step of glycolysis is the hexokinase reaction or a reaction preceding it such as the penetration of glucose into the cell. This is the initiating reaction; the rates of the following reactions do not depend on the amounts of enzyme, but on the amounts of substrate supplied by the preceding steps.

McIlwain: One has to be careful in the use of the term "excess", particularly if one implies unutilized excess; as has been pointed out, the intermediates often do not accumulate. I feel that what may seem to be unutilized excess in potential enzyme activity is very important in maintaining an ordered sequence of reactions, such as the tricarboxylic acid cycle. In the cycle in a given tissue one may have certain stages with enzyme capacities 50 times those of others. This is probably concerned with keeping the cycle functioning as a cycle; for in comparing the levels of such enzymes e.g. in nervous tissue, one concludes that it is the particular stages of the cycle where there is the possibility of diverging from it, that certain enzymes appear at high activity in comparison with the others. Thus, the high activity can be related to the functioning of the tissue.

I have also been impressed by the extent to which the total level of glycolytic systems in tissues from the central nervous system can give approximately the same yield of energy-rich phosphate as the oxidative series. Sometimes the glycolytic series may have enzyme levels of the order of 20–30 times those of steps of the oxidative series. It appears to be a real alternative, though normally functioning only briefly at maximum rate, and the high level of enzyme activity is necessary to make the anaerobic reaction a real alternative.

Krebs: Does this apply also to hexokinase? That is the critical point. When I said "in excess" I meant that more can be dealt with by these enzymes than can be supplied by the initial step.

McIlwain: The rate of hexokinase in, say, cerebral tissues, is approximately ten times that of the recorded rates of the initial stages of the tricarboxylic acid cycle, so that the rate at which hexokinase operates is more akin to the level of glycolytic enzymes.

Hinshelwood: That is under the condition of an *in vitro* test. Are they in these large excesses under the conditions of formation in the tissue? The proportion of enzymes would be determined by the optimum conditions of the original growth and formation of these enzymes in the tissues, not by the level of their amount of activity in an *in vitro* test, which is an artificially established condition.

McIlwain: A relevant point is that the high activity of the enzymes themselves keeps their intermediate substrates at such relatively low levels; and that, I feel, is part of the mechanism of integration of their individual reactions to a cycle.

Siekevitz: On the hexokinase point, LePage has done experiments in which he compared normal and tumour liver, and he found that in the normal liver hexose diphosphate was a much better substrate for glycolysis than glucose, and he came to the conclusion that hexokinase is limiting (LePage, G. A. (1950). *Cancer Res.*, **10**, 77).

Krebs: Many experiences have shown that hexokinase is limiting.

Chance: But some of our data indicate that hexokinase can produce ADP much faster than the mitochondria can utilize it.

Krebs: Is that in muscle?

Chance: No, that is in yeast cells and ascites tumour cells under special conditions of a metabolic transient.

Lipmann: We talk as if we were dealing with a homogeneous system, whereas we are dealing with a highly heterogeneous system. We cannot just ignore that and speak as if we could set up general conditions where every substrate meets another one, and meets a catalyst uninhibited by membranes or permeability barriers. It would be more useful to think in terms of heterogeneity rather than in terms of homogeneity. We forget that so easily.

ON THE MEANING OF INTRACELLULAR STRUCTURE FOR METABOLIC REGULATION

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This presentation deals with some of the facts of the existing cytological findings on cell structure and with the biochemical inferences which may be deduced from them. As such, it will abide within the spirit of this symposium, which has to do not with the recitation of the old, nor even of the new, but with the contemplation of the foreseeable.

By now, I hardly dare suggest that the cell is not a bag, but I do think that most biochemists do not fully realize to what extent the cell is made up of more or less distinct compartments. Due mostly to the pioneering work of Porter and Palade in America and Sjöstrand and his group in Sweden (cf. Sjöstrand, 1956), we know that the cytoplasm is divided into numerous compartments, which include the nucleus, the mitochondria, various inclusion bodies in different cells, and the endoplasmic reticulum. Of these, the latter is least understood, and therefore, considering the nature of this symposium, will be the one discussed in the present paper. This system, and some physiological implications which can be deduced from its existence, have been described in a recent excellent review by Palade (1956*a*). I propose to go on from there, bringing in some biochemical concepts to fulfil the intimations previously expressed.

It is agreed among the majority of cytologists that the endoplasmic reticulum (ER) is a system of lumina limited by a membrane (about 75 Å thick) and taking the forms of canals, vesicles and lacunae or cisternae within the cell. These spaces are more or less interconnected among themselves and form a continuum which permeates the cytoplasm

of nearly all cells (Palade and Porter, 1954; Palade, 1956*a* and *b*; Porter, 1955, 1957; Porter and Palade, 1957). Since this system is bounded by a membrane we can speak of an intraluminal space and of a periluminal (cytoplasmic matrix) space. This structure is not uniform throughout a particular cell but it can be visually differentiated into local variations. In some parts of the cell the membranes of this arrangement are naked, while in other parts they are covered by ribonucleoprotein particles (about 150 Å in diameter). In various regions of the cell the canals of the system are seemingly rigidly disposed in relation to each other, while in other parts there is a helter-skelter, disordered ordination of the vesicles. In the pancreatic acinar cell (Fig. 1) the orderly arrangement takes the form of a system of parallel canals (cisternae) with particles on their membranes and with a uniformly repeating distance between them, while even in the same cell there is a region, the Golgi zone, where the membranes are bare and are disposed in stacks and swarms of vesicles (Fig. 2). In the muscle cell we find a distinctive, lace-like network surrounding the fibrils (Porter and Palade, 1957). The visualization of the ER in many other cell types can be attained by glancing through the references mentioned above. On the basis of a unitary hypothesis, there is very little reason to doubt that the local variations within a single cell, or the variations in character between different cells, are just variations, and that all are derived from a common system of membrane-limited lumina (Palade, 1956*a*; Porter, 1957). This system fragments upon homogenization and gives rise to the microsome fraction in the usual differential centrifugation technique (Palade and Siekevitz, 1956*a*).

Now I propose to take you on a brief journey from the outer spaces of the extracellular fluid to the inner ones of the nucleus. Several workers have demonstrated that the cell membrane in most cells is at some places invaginated to form inpocketings into the cell (Palade, 1956*a*; Parks and Chiquoine, 1956; Palay, 1958). Figures 3, 4 and 5 show these invaginations as they occur in a spleen macrophage, in a

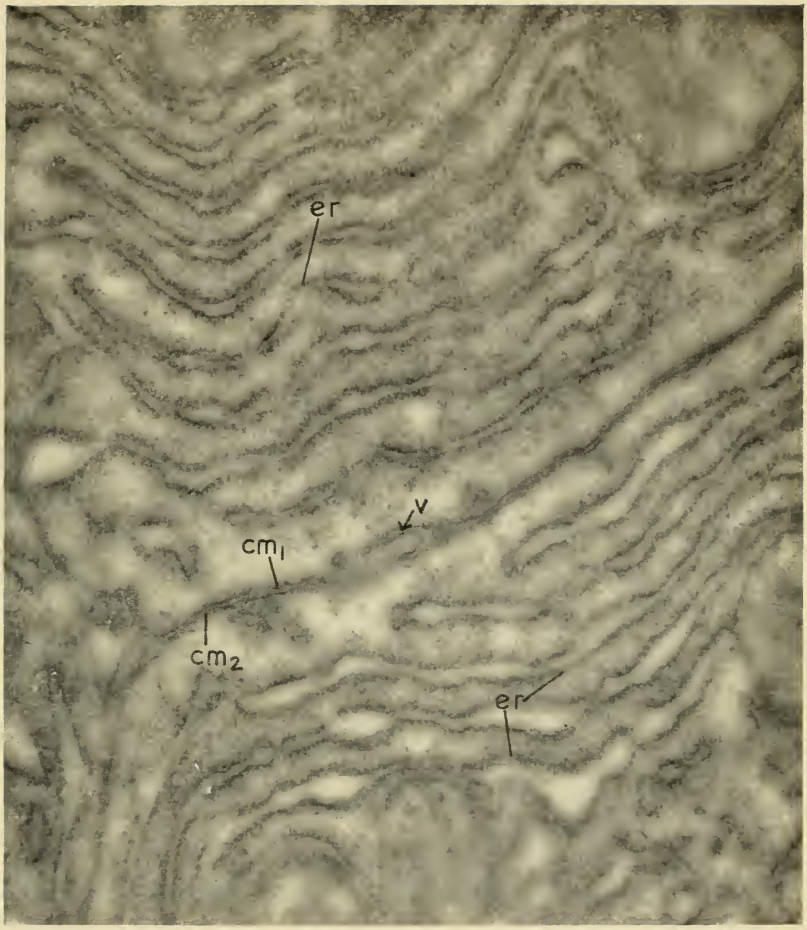

FIG. 1. Junction between two acinar cells of guinea pig pancreas. cm_1 and cm_2 = cell membranes of two adjacent cells; v = vesiculation of one cell membrane; er = endoplasmic reticulum.
Magnification = 33,333. (Palade and Siekevitz, 1956*b*. Reproduced by permission of the Editors, *J. biophys. biochem. Cytol.*)

[*facing page 18*

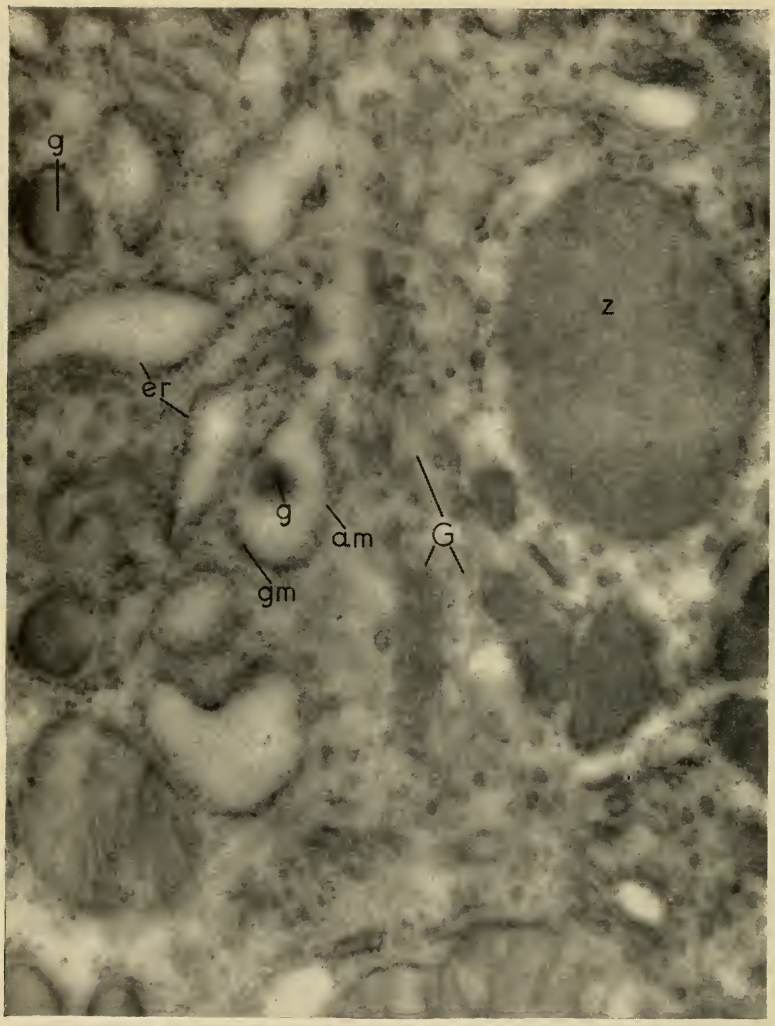

FIG. 2. Periphery of the Golgi zone (centrosphere region) in acinar cell of pancreas. er = endoplasmic reticulum (ER); gm = granular membranes of the ER having ribonucleoprotein particles; am = agranular membranes of the ER having no particles and in continuity with the granular membranes at this point; G = Golgi region showing stacks and swarms of bare membranes; g = intracisternal granule inside cisterna of the ER; Z = zymogen granule.
Magnification = 43,333. (Kindly loaned by G. E. Palade, unpublished.)

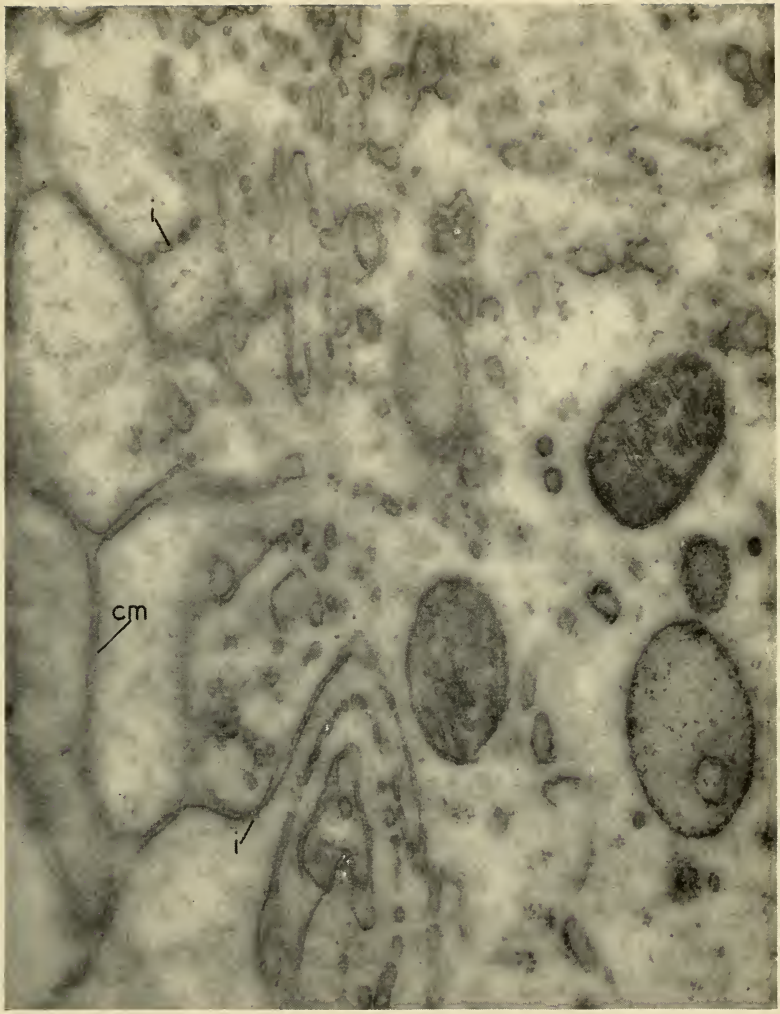

FIG. 3. Cell periphery of macrophage in the spleen. cm = cell membrane; i = invagination of the cell membrane which goes deeply into interior of the cell.

Magnification = 36,666. (Palade, 1956*a*. Reproduced by permission of the Editors, *J. biophys. biochem. Cytol.*)

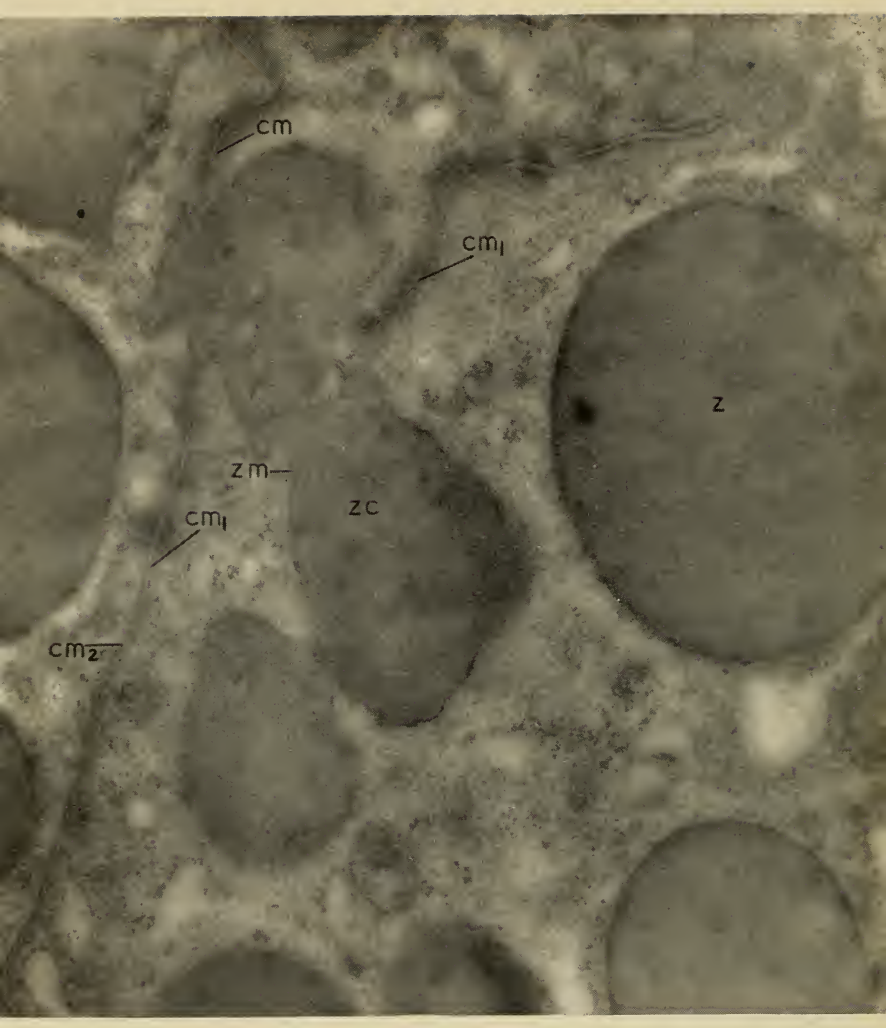

FIG. 4. Apical pole of acinar cell of guinea pig pancreas. z = zymogen granule; zc = zymogen content which is already in lumen of pancreatic duct; cm_1 and cm_2 = cell membranes of adjacent cells which are in continuity with membrane (zm) of zymogen granule.
Magnification = 40,000. (Kindly loaned by G. E. Palade, unpublished.)

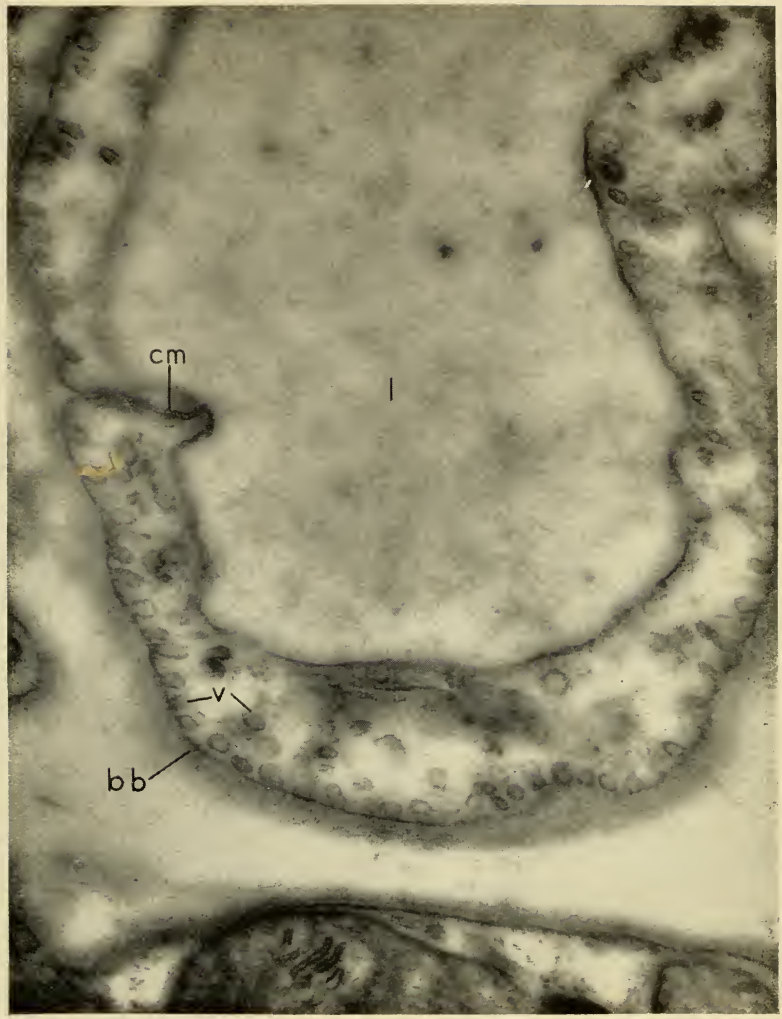

FIG. 5. Endothelial cell of a blood capillary (rat heart muscle). l = lumen of capillary; cm = cell membrane; bb = brush border: v = vesicles inside cell which are in many cases invaginations of cell membranes. Magnification = 43,333. (Palade, 1956*a*. Reproduced by permission of the Editors, *J. biophys. biochem. Cytol.*)

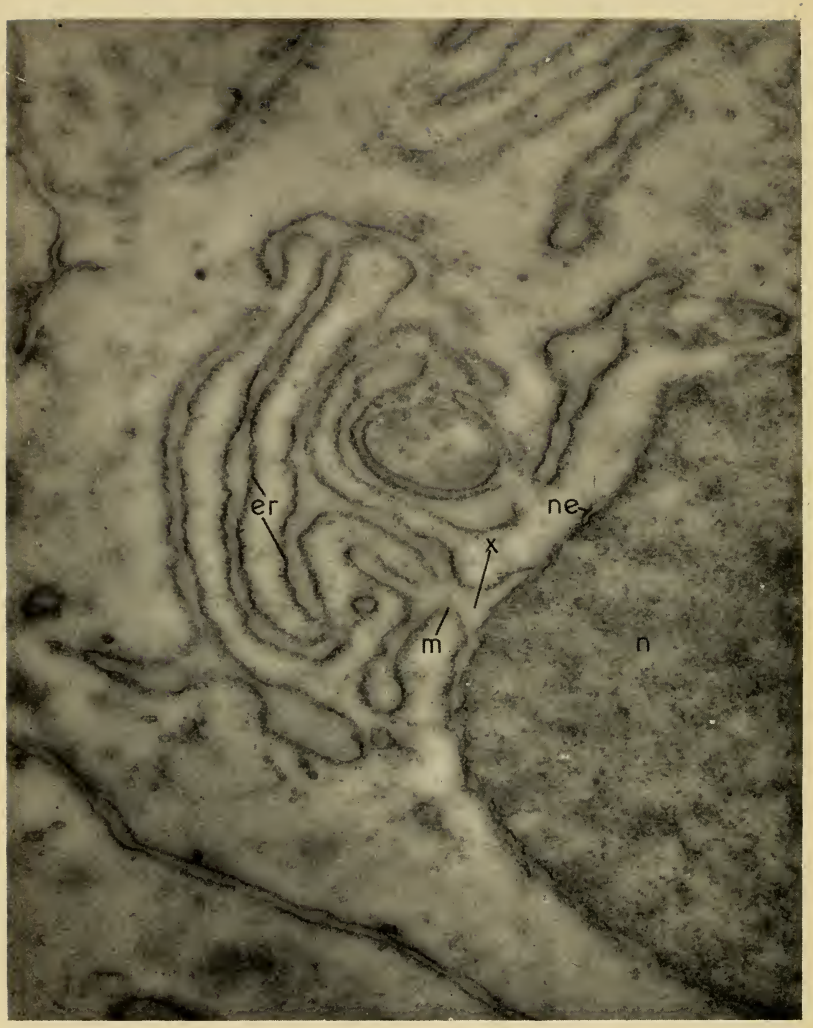

FIG. 6. Portion of a notochord cell in Amblystoma larvae. n = nucleus; ne = nuclear envelope consisting of two membranes and a perinuclear space in between; er = endoplasmic reticulum; m = membrane of ER which is continuous with outer membrane of nuclear envelope so that the perinuclear space is continuous with cavities of ER at spot marked "X". Magnification = 26,666. (Kindly loaned by K. R. Porter, unpublished.)

pancreatic acinar cell, and in the endothelial cell of a blood capillary. In some cases, as in the macrophages, these infoldings reach deeply into the cell, and it is thought that the probing tip gets broken off, that the membrane reforms around it, and that the fluid which is thus enclosed and which came from the extracellular space is now within the cell, enclosed within a membrane-bounded vesicle. Epstein (1957) thinks that in the Rous sarcoma ascites cell the membranes of such infoldings are catenated with the membranes of the ER and thus the extracellular fluid is continuous with the fluid in the intraluminal spaces; but this point is still under investigation. However, all workers agree that the invaginations of the cell membrane do exist, and the physiological meaning of this existence is discussed below (p. 20).

When sections of cells from various tissues are examined with the electron microscope, it is observed, particularly in the liver cell, that many of the mitochondria seem to be enclosed in folds or caps formed by the curving around of some of the membranes of the ER. Only a small distance thus remains between one of the ER membranes and the outer mitochondrial membrane. Bernhard and Rouiller (1956) have made a detailed study of this relationship and believe that the close structural apposition portends a close functional relationship between the mitochondria and the ER. It should be understood that the mitochondria do not sit in the intraluminal cavities of the ER, but lie outside, in the cytoplasmic matrix.

As we go inward and reach the nucleus, we come upon another sort of relationship. This link has been amply shown (Watson, 1955; Epstein, 1957; Palade, 1956*a*) particularly by Watson, who has come to the following generally accepted conclusion: cells from many tissues have nuclei which are bounded by two successive membranes. Upon closer examination it was noted that the inner membrane curves back to merge with the outer one and is continuous with it, and that the inner one is devoid of particles, while the outer one is clearly a membrane of the ER and can be identified as such

by the presence of the typical particles on its surface and by its continuity with other ER membranes (Fig. 6). Thus, what at first glance was thought to be a double nuclear membrane turns out to be an extension of the membrane of the ER. The space between these two membranes, the perinuclear space, is continuous with the intraluminal space between the membranes of the ER and is, in fact, just an extension of it. Thus we can say that, unlike the mitochondria, the nucleus is closely surrounded by the cavities of the ER, with only one membrane separating the contents of the nucleus and the contents of the intracisternal space. Watson (1954, 1955) has also shown the existence of pores, which are actually windows through the perinuclear space, so that the contents of the nucleus are continuous with the contents of the cytoplasmic matrix (cf. Fig. 7).

What are the physiological implications of this brief representation of the cell? It has been accepted for a long time that the passage of molecules into and out of the cell occurs solely by a discrete individual passage of compounds through a fixed cell membrane, either by a carrier mechanism or by passage through pores or by "solubilization" in a lipoidal phase. Nowadays, we are beginning to understand that in all cells, with the probable exception of the mature mammalian red blood cell, another process also occurs, and probably simultaneously with the first. This second process is emphasized in Fig. 7, which shows a stylized and idealized representation of that most common of cells, the typical cell. The extracellular fluid is shown to be brought into the cell, in part, by means of actual movements of the membranes. Whether the vesicles so formed are broken down and the contents released into the general cytoplasmic matrix—and this is the accepted view—or whether these vesicles finally communicate with the vesicular spaces of the ER, or whether both processes take place, is not known. Nevertheless, it is quite clear that by this mechanism the membranous surface of the cell is multiplied many times and what is probably more important, the extracellular fluids are brought into the

innermost reaches of the cytoplasmic matrix. On this basis, perhaps the mitochondria are not so far removed from the compounds of the blood, including oxygen, as was formerly thought. On the other hand, there is good reason to believe

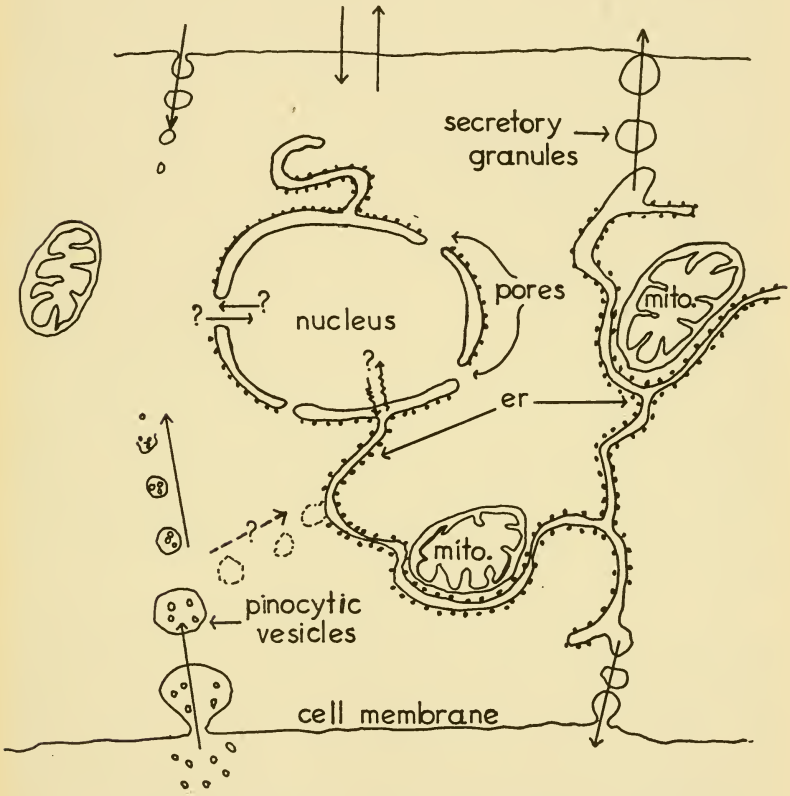

FIG. 7. Stylized representation of a cell. Description given in text.

that the extensive system of intraluminal spaces in some cells can act as a secretory device where, finally, the contents within these spaces are secreted into ducts or into the blood by a process of fusion of the membranes of the ER with the membrane lining the cell. The close disposition of the

mitochondria with the ER could facilitate the removal of mitochondrial products, including carbon dioxide, to the outside through this mechanism; in other words, the vesicular systems of the cell are not static entities but behave as intrusion and extrusion machines. It can be proposed that the regulation of the input and the output of the cell, which was formerly thought to be due only to the active metabolism of a spatially fixed cell membrane, is now thought to be due also to an extensive system of membranes which move within the cell and which fuse with the cell membrane and are really adjuncts of the cell membrane. Palade (1953) and Bennett (1956) have pictured this concept, even with the idea that the membranes are not passive in this regard but actively flow and perhaps pump materials through the intracisternal spaces, either from outside the cell inwards via the pinocytic vesicles, or from inwards to the outside by means of that part of the agranular reticulum which is connected with the granular reticulum of the ER. Bennett (1956) also postulates that there are specific binding sites on these membranes to account for the selectivity of compounds moving in and out of the cell. Palay (1958) has given a detailed description of the relationship between the extracellular space and the intracisternal spaces of the ER and has postulated a similar theory of secretion based on his morphological observations.

Now what does all this mean, first in terms of permeability? We can all immediately understand that the old concept of intracellular space and extracellular space has to be refined (cf. Palade, 1956*a*). Most of the experiments having to do with permeability, by measuring the uptake or output of compounds, be they charged ions or uncharged organic compounds, will now have to be re-examined. For is not the fluid inside the pinocytic vesicles still extracellular, the compounds within still having to pass a membrane, either passively, actively, or by membrane dissolution, before they can mix with compounds in the cytoplasmic matrix? And conversely, is it not possible that compounds within the secretory granules—which are later shown to be formed from

the vesicular structures of the ER—are effectively out of the range of intracellular enzymes? In other words, at the present moment we are in confusion when we speak of intracellular and extracellular, and, in truth, the descriptive value of these terms has been greatly negated by the recent morphological data and hence have no true meaning in a physiological sense.

A more realistic picture of the way compounds get in and out of the cell may be the following. Firstly, their importation: undoubtedly, the cell concentrates and secretes molecules by passage through the cell membrane, by some as yet unknown means. However, it is now recognized that pinocytosis, the bringing in of extracellular material by an engulfing or invaginating mechanism of the cell membrane (Lewis, 1935–36), is also a property of many cells. It has been beautifully shown by Holter and his colleagues in the uptake of various proteins, glucose and methionine by amoeba (Holter and Marshall, 1954; Chapman-Andressen and Holter, 1955), and at an electron microscopic level by Parks and Chiquoine (1956) in the uptake of colloidal gold by phagocytes, by Wissig and Palade (personal communication) in the uptake of ferritin particles by capillaries (Fig. 8) and by Odor (1956) in the uptake of thorium dioxide by the mesothelium of the mesentery. The meaning of the implications derived from these observations is obvious, and some biochemical ideas based on these premises are developed below (p. 34).

Similarly, the mechanism of secretion of intracellularly formed compounds should also be looked at in this new light. Palay (1958) and Palade and the present author (Siekevitz and Palade, 1958*a*, *b* and *c*) have looked into the secretion and prior formation of the zymogen granules of the pancreas, and in these papers there is a good deal of morphological and biochemical evidence that the secretory enzymes are synthesized at the site of the ribonucleoprotein (RNP) particles attached to the ER membrane, that these enzymes are then somehow pushed into the cavities, the intracisternal (luminal) space, of the ER, and are then transported to the Golgi region of the cell to be there packaged into the mature

zymogen granules. By the coalescence of the membranes surrounding the zymogen granules with the cell membrane, the contents of the granules are pushed out into the pancreatic ducts. Fig. 4 shows such a latter event in the process of happening, while Fig. 9 shows the presence of the newly synthesized secretory enzymes within the cavities of the ER of the guinea pig pancreas, where they form intracisternal granules. We can isolate these intracisternal granules and have shown that they contain the same enzymes, and in the same concentrations, as they are found in the isolated zymogen granules (Siekevitz and Palade, 1958*b*). Whether this transporting process is peculiar to the pancreas, or is found in all other tissues, like the glands or even the liver, which secrete substances to the various extracellular fluids, is not known, but Palay (1958) thinks it could well be a general process of all cells. But more about this speculation later on. Incidentally, it might well be fortuitous that the microsomes have become linked with protein synthesis in the minds of biochemists; most of the work has been done with liver and with pancreas, and these are the organs *par excellence* which secrete proteins. It could well be that ribonucleoprotein particles are implicated in protein synthesis but in the liver and pancreas these structures are attached to the ER membranes, and the high rate of protein turnover of the microsomes is merely a reflection of the high rate of synthesis of secretory proteins by those organs, the synthesized protein being then secreted via the ER (cf. Howatson and Ham, 1957).

I have given a picture of what I believe to be the present state of knowledge of the problem of the passage of molecules into and out of the cell. Most of the initial examples have dealt primarily, and necessarily, with large molecules, but does the same viewpoint hold for smaller molecules, such as the amino acids or glucose? We do not know, but let us consider some schemes of various restricted parts of cellular metabolism and see where it will lead us. Of course, this will also have to do with the segregation of enzymes within the cell; and mention should first be made of some observations

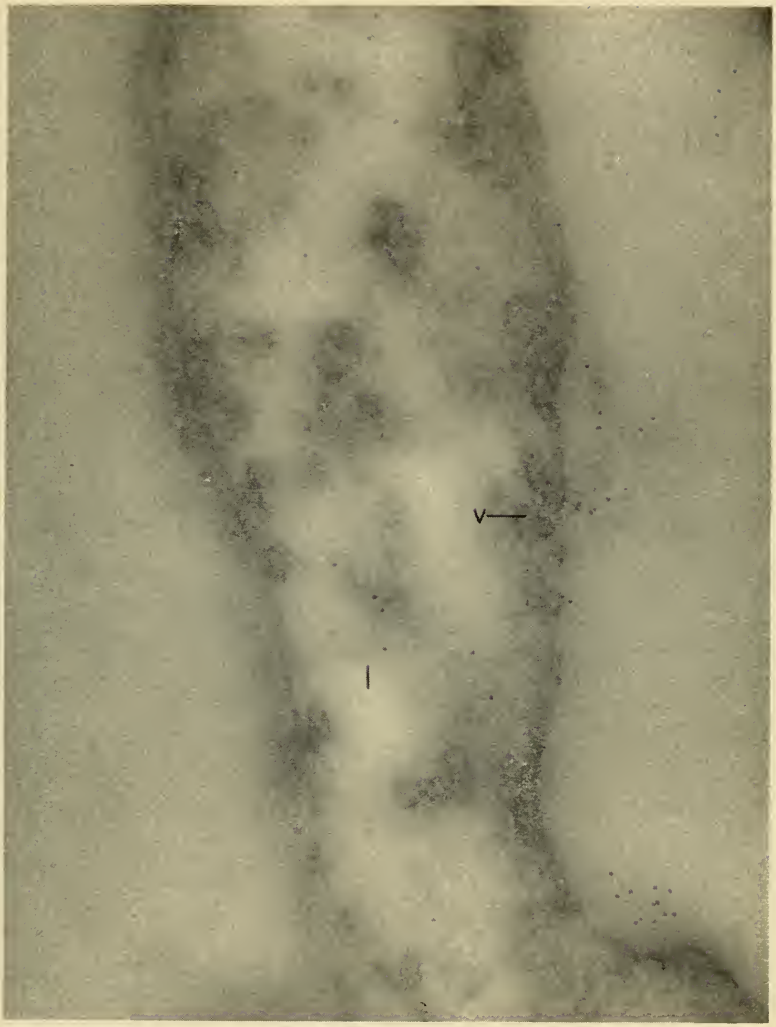

FIG. 8. Uptake of ferritin particles by an endothelial cell in a blood capillary (rat heart muscle). Cf. Fig. 5. l = lumen of cell; v = vesiculations. The small dense dots are ferritin particles, many of which can be seen inside the vesicles.
Magnification = 90,000. (Kindly loaned by S. Wissig and G. E. Palade, unpublished.)

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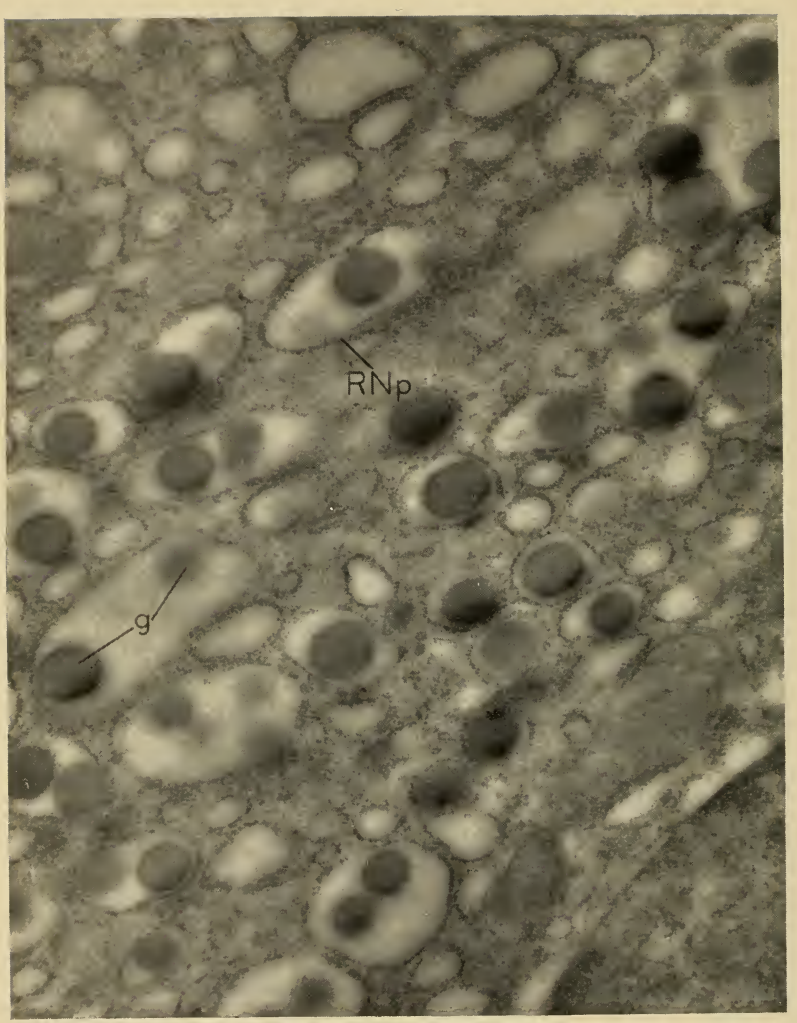

FIG. 9. Intracisternal granules in the basal region of an acinar cell of guinea pig pancreas. g = intracisternal granules inside vesicles of the ER. These vesicles are bounded by membranes having attached ribonucleoprotein particles (RN_p). Cf. Fig. 2.
Magnification = 26,666. (Palade and Siekevitz, 1956*b*. Reproduced by permission of the Editors, *J. biophys. biochem. Cytol.*)

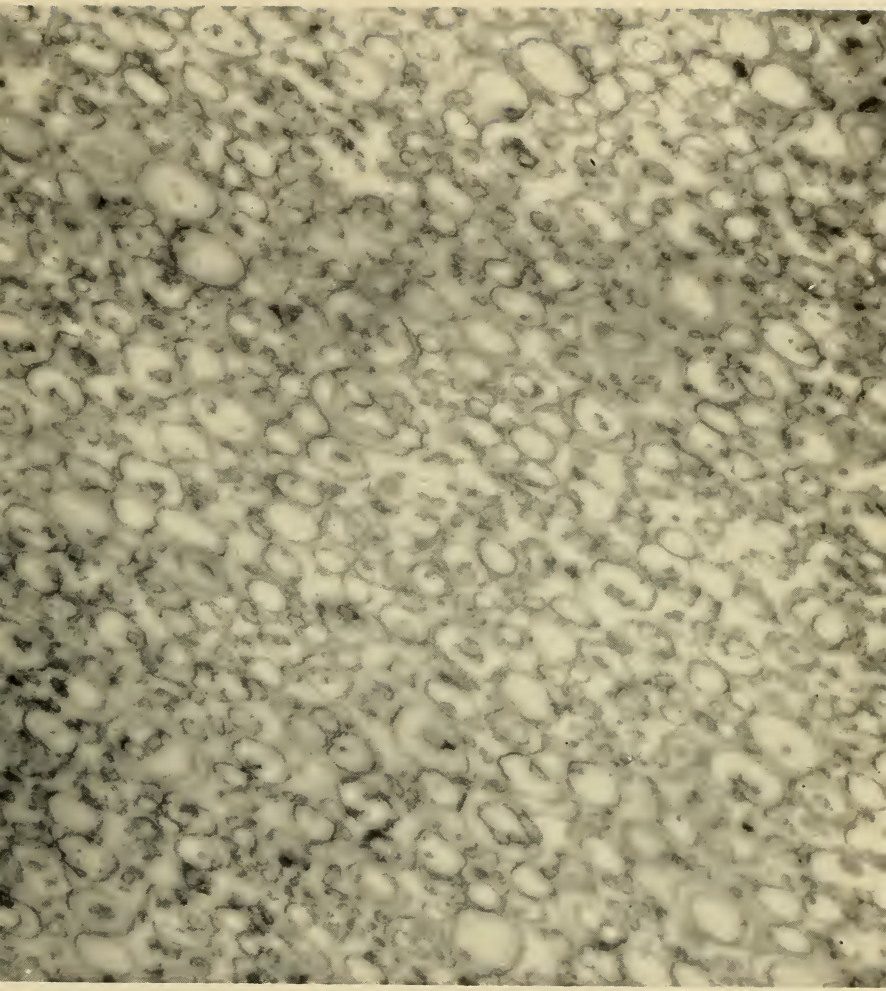

FIG. 10. Mitochondrial membrane pellet. This pellet was obtained by centrifuging a DOC-treated mitochondrial suspension as described by Watson and Siekevitz (1956). There are no intact mitochondria, only single- and double-membraned vesicles, some much smaller than and some nearly as large as mitochondria. The mitochondrial matrix is gone, leaving optically-empty vesicular contents.
Magnification = 20,000. (Watson and Siekevitz, 1956. Reproduced by permission of the Editors, *J. biophys. biochem. Cytol.*)

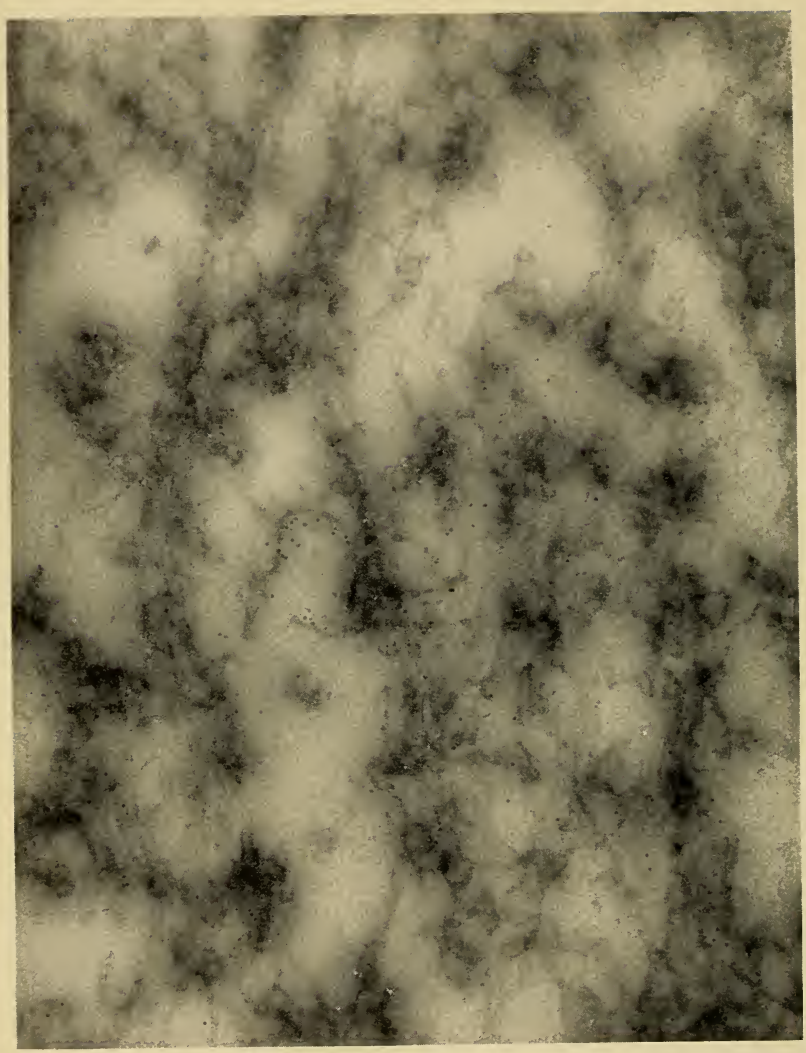

FIG. 11. Microsomal membrane pellet. This pellet was obtained as described in the text. No attached ribonucleoprotein particles are visible and the membranes form only a few of the type of vesicles which are found in intact microsomes.
Magnification = 63,333. (Kindly loaned by G. E. Palade, from Ernster, Palade and Siekevitz, unpublished.)

on the intracellular membranes, the mitochondrial membrane and the membranes of the ER, for it is evident that concomitantly with the divisions which they produce in the cell these membranes can be important regulators of cellular metabolism.

After a good many years, it was finally shown (Watson and Siekevitz, 1956; Siekevitz and Watson, 1956) what a good many biochemists had long suspected (cf. references in Siekevitz and Watson, 1956): that the succinoxidase system and most, if not all, of the electron transport system of the mitochondria constituted a structural part of the membrane. Fig. 10 shows such a mitochondrial membrane preparation and Table I gives the relevant data as to the concentrations of phospholipid, succinoxidase, and cytochrome oxidase in this membrane fraction. Together with previous results, these latest ones should leave no doubt concerning the intimate localization of these enzymes.

Table I

SOME CHEMICAL AND ENZYMIC PROPERTIES OF MITOCHONDRIA AND MITOCHONDRIAL MEMBRANES

	mg. Protein/g. liver	*mg. Phospholipid/g. liver*	*mg. Phospholipid/mg. protein*
Mitochondria	9·50	2·12	0·22
Mitochondrial membrane	0·56	0·33	0·59

	Succinoxidase		*Cytochr. oxid.*		*DPNH-cyt. c red.*	*Adenyl. kinase*
	Act.	*Sp. act.*	*Act.*	*Sp. act.*	*Act.*	*Act.*
Mitochondria	1320	868	233	153	492	90·0
Mitochondrial membrane	498	5550	79	878	0	0

Data from Siekevitz and Watson (1956).

Now in the last several months, Dr. Ernster of the Wenner-Grens Institute, Stockholm, working in our laboratory, has succeeded in isolating microsomal membranes, or rather, in reforming membranes. Palade and the present author (Palade and Siekevitz, 1956*a*) had previously found that liver

microsomes could be dismantled with 0·3 per cent deoxycholate (DOC) so as to render "soluble" their reduced diphosphopyridine nucleotide (DPNH)-cytochrome *c* reductase activity. What is meant by this was that if the disintegrated microsomal suspension were spun down for two hours at 105,000 ***g*** to bring down the ribonucleoprotein particles, this enzyme remained in the supernatant solution. What Ernster did was to take this supernatant fraction which contained all of the microsomal enzyme, albeit somewhat inactivated, and effectively get rid of the DOC by diluting the supernatant solution some five- to ten-fold with a sucrose solution. As a control he had one specimen to which was added the same amount of sucrose solution but containing the same concentration of DOC as was used originally to disintegrate the microsomes. After spinning at 105,000 ***g*** for two, or six, or sixteen hours, he found that he obtained a large pellet from the sucrose-diluted supernatant but only a very small pellet from the sucrose and DOC-diluted supernatant. Palade took a picture of this pellet from the sucrose-diluted supernatant and, as Fig. 11 shows, found that a specimen of microsomal membranes had been obtained, minus their formerly attached nucleoprotein particles, but which was disorganized in that only a few typical microsomal vesicles could be seen. At present we take the view that the membranes were initially truly solubilized, perhaps by being spread out in a thin film in the presence of the detergent, and by lowering the concentration of the detergent, they recoiled upon themselves but not in their original configuration, and became responsive to being sedimented. Tables II and III give some representative data on the properties of this microsomal enzyme and on the enzyme of the microsomal membrane fraction. With regard to the variations in response of the microsomal DPNH-cytochrome *c* reductase to treatment: briefly, sometimes we obtained microsomes with high enzymic activity, and if let stand in sucrose alone, or more so in sucrose-DOC solution, they lost much of their activity. Sometimes, the original microsomes had low activity, but could be activated by stand-

ing in sucrose alone, or more so in sucrose-DOC solution (Table II). This latter activated suspension, if let stand or if diluted with sucrose, lost enzymic activity rapidly, and it was from

Table II

EFFECTS OF VARIOUS TREATMENTS ON DPNH-CYTOCHROME *c* REDUCTASE AND TPNH-CYTOCHROME *c* REDUCTASE ACTIVITIES OF RAT LIVER MICROSOMES

The undiluted microsome suspension contained microsomes from 214 mg. wet weight liver in Expt. 8 and 200 mg. wet weight liver in Expt. 13. All centrifugations were performed at 105,000 *g*. DOC = deoxycholate, pH 7·5–7·8.

			μmoles oxid./min./g. liver	*PNH*
Expt. No.	*Treatment*	*Fraction tested*	*DPNH*	*TPNH*
8	In sucrose, fresh	Microsomes	30·6	—
	In sucrose, kept 6 hr. at 0°C	Microsomes	57·6	—
	In 0·26% DOC, fresh	Microsomes	81·6	—
	In 0·26% DOC, kept 3 hr. at 0°C	Microsomes	61·5	—
	In 0·26% DOC, kept 6 hr. at 0°C	Microsomes	47·4	—
	In 0·26% DOC, then diluted 2·5 ×, kept 6 hr. at 0°C	Microsomes	28·3	—
	In 0·26% DOC, then diluted 5 ×, kept 6 hr. at 0°C	Microsomes	16·4	—
	In 0·26% DOC, then diluted 10 ×, kept 6 hr. at 0°C	Microsomes	14·2	—
	In 0·026% DOC, kept 6 hr. at 0°C	Microsomes	74·5	—
13	In sucrose, fresh	Microsomes	58·5	0·86
	In sucrose, kept 8 hr. at 0°C	Microsomes	27·9	0·99
	Treated with 0·26% DOC, then centrifuged 2 hr.	RNP particles	0·7	0·00
	Supernatant from above, diluted 5 ×, kept 6 hr. at 0°C	Supernatant from above	15·6	0·93
	Above, centrifuged 6 hr.	Membrane pellet	8·6	0·19
	Above, centrifuged 6 hr.	Supernatant from above	2·7	0·58

Data taken from Ernster, Palade and Siekevitz (unpublished).

this suspension that we obtained our microsomal membrane fraction. Because of these responses, dependent on the chosen base-line, on the activity of the original microsomes, or upon that of the activated microsomes, or upon that of the diluted low-activity microsomes, we obtained a wide range of recovery

Table III

COMPARISON OF CYTOCHROME *c* REDUCTASE ACTIVITIES OF MICROSOMES AND MICROSOMAL MEMBRANES

		Membrane pellet			*Fresh microsomes*		
Expt. No.	*Fraction tested*§	*DPNH-cyt. c red. act.**	*DPNH-cyt. c red. sp. act.*†	*TPNH-cyt. c red. act.**	*DPNH-cyt. c red. act.**	*DPNH-cyt. c red. sp. act.*†	*TPNH-cyt. c red. act.**
2	0–2 hr.	2·03‡	2·60	—	10·35	—	1·05
3	0–2 hr.	2·01	2·95	—	10·10	—	0·54
	2–6 hr.	2·19	2·72	—	—	—	—
4	0–2 hr.	1·77	2·80	0·13	5·05	1·73	0·80
	2–13 hr.	1·36	2·13	0·19	—	—	—
	Supernatant from 13 hr.	0·38	—	0·21	—	—	—
6	0–16 hr.	4·80	3·25	0·62	16·20	6·86	1·50
	Supernatant from 16 hr.	0·0	—	0·29	—	—	—

* μmoles DPNH or TPNH oxid./min./g. liver.
† Act./mg. protein N.
‡ When resuspended in 0·12% DOC = 2·55; in 0·24% DOC = 5·11; in 0·33% DOC = 4·86.
§ No. refers to length of spin at 105,000 *g* used to bring down pellet from DOC-treated microsomes after previously spinning down RNP particles. Thus 0–2 hr. = 2 hr. pellet and 2–6 hr. = pellet obtained after spinning supernatant from 0–2 hr. pellet for 4 hrs.

Data taken from Ernster, Palade and Siekevitz (unpublished).

figures. However, it is clear that we are recovering a good deal of the initial enzyme activity in our preparation. As Table III shows, no matter what the enzyme activity of the initial starting materials, the membrane pellets all had very similar enzymic specific activities. Also, no matter for how long we spun the diluted supernatant, the resulting pellets, while differing in amount, all had the same specific enzyme activity and all looked the same in the electron micrographs. All of the active enzyme which we recover is a part of a structure which we can identify as the membranes of the microsomes. The reduced triphosphopyridine nucleotide (TPNH)-cytochrome *c* reductase activity behaves quite differently from the DPNH enzyme in its response to treatment (Table II) and in the fact that it comes down as a pellet much more slowly during the centrifugation (Table III). We believe that the membrane material having this activity

is reformed into much smaller units than is the membrane material having the DPNH-cytochrome *c* reductase activity.

Here, then, are the two presently known electron-transport chains of the cell, and both seem to be an intimate part of a membrane structure, one in the mitochondria, and one in the microsomes. We have given the possible significance of this localization in the case of the mitochondrial enzymes (Siekevitz and Watson, 1956). I would suggest that one of the functions of the microsomal enzyme may be in transporting ions across the membrane, in a manner similar to that visualized by Conway (1951) by the use of a redox pump scheme. Conway's scheme could be used to explain the active transport of hydrogen ions, of cations, and with modifications, of anions, across membranes. Its attractiveness lies in the fact that the active carrier, being a reduced, metal-containing catalyst, binds the cation and is the same system as the energy-providing system. Its particular enticement in the morphological sense lies in that not only does it extend the idea of the ER system being an adjunct of an active cell membrane system, but that it could also provide for intracellular differences in ion concentrations and for pH variations deep within the cell. It is clear what this would mean for enzymic activities. Furthermore, the ER system not only divides the cell by its magnificent virtue of being, but also, if our aforementioned ideas are correct, by acting enzymically in such a way as to produce differences in ion concentrations between the two sides of the membranes and perhaps also differences between localities contiguous and remote from the membranes. In this general connexion, it has already been suggested (Porter and Palade, 1957) that the ER in the muscle cell might act as an internal conducting system, the membranes serving as conductors of the excitatory impulse. The idea of a flavoprotein-mediated electron-transport and cation-carrier system being a part of the ER membrane certainly does no harm to this concept.

Concerning the phospholipid composition of membranes, while it is true that the mitochondrial membranes isolated

previously (Siekevitz and Watson, 1956) were concentrated in phospholipid over the original mitochondria, the same cannot be said definitely about the microsomal membrane preparation, for we could not find any concentration in phospholipid in the microsomal membranes over the original microsomes. In the case of the liver, the isolated microsomes have a higher concentration of phospholipid than the original homogenate (Palade and Siekevitz, 1956*a*), but this is not the case with the pancreas (Palade and Siekevitz, 1956*b*). For these reasons, the mitochondrial membranes may be regarded as being lipoprotein in nature, and the microsomal membranes, the membranes of the ER, as being protein membranes. These differences in composition might explain the differences in manipulation which are necessary to obtain the membranes from mitochondria (Watson and Siekevitz, 1956) and from microsomes (cf. above, p. 25). Also, as can be seen from Table III, the mitochondrial DPNH-cytochrome *c* reductase was solubilized, like the microsomal enzyme, by DOC. But when we tried diluting this supernatant with sucrose, in the same way as we did for the DOC-treated microsomes, nothing appeared upon recentrifugation. Since most of the mitochondrial cytochrome oxidase and succinoxidase had already been centrifuged down as membranes, we are at a loss to decide whether the mitochondrial DPNH-cytochrome *c* reductase is not a membrane enzyme, or whether it is and is so damaged that it cannot be reformed.

Mention having been made of the identification of certain enzymes with membranes, a phenomenon to which we have alluded before (Siekevitz and Potter, 1955) is now described in more detail. First, Table IV shows the peculiar results of what at first glance should be a rather straightforward experiment. As an increasing amount of hexokinase is added to a medium containing adenosine triphosphate (ATP) and substrate together with mitochondria, we obtain the familiar picture of the increasing inorganic phosphate (P_i) uptake into glucose-6-phosphate (G-6-P) and an increasing oxygen uptake due to the well known phosphate acceptor effect. However,

Table IV

EFFECT OF ADDED HEXOKINASE ON NUCLEOTIDE LEVELS IN A MEDIUM IN WHICH MITOCHONDRIA ARE UNDERGOING OXIDATIVE PHOSPHORYLATION

ml. Hexokinase added	*μl. O_2 uptake*	*μM P_i uptake*	*In medium*		
			μM ATP	*μM AMP*	*μM ADP*
0	37	2	2·6	0·0	0·3
0·005	36	1	2·6	0·0	0·4
0·01	37	8	2·6	0·0	0·3
0·02	46	14	2·6	0·0	0·3
0·04	54	23	2·5	0·1	0·3
0·06	74	25	1·7	0·3	1·0
0·08	76	24	0·8	0·7	1·1
0·10	75	23	0·5	1·2	1·1

Data taken from Siekevitz and Potter (1953).

at these early points of increases we notice that the ATP in the medium does not change in concentration. Either the hexokinase which we add to the medium is not acting on the ATP there, or, if it is, the adenosine diphosphate (ADP) resulting goes into the mitochondria, is phosphorylated there to ATP, and the ATP is secreted rapidly to maintain the ATP concentration in the medium. Which is it? In Table V are the results

Table V

EFFECT OF ADDED HEXOKINASE ON CONCENTRATIONS AND SPECIFIC RADIOACTIVITIES (SA) OF INTRAMITOCHONDRIAL AND EXTRAMITOCHONDRIAL NUCLEOTIDES AND GLUCOSE-6-PHOSPHATE IN A MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION SYSTEM

	Intramitochondrial			*Extramitochondrial*		
	0 *Hex.*	2 *Hex.*	4 *Hex.*	0 *Hex.*	2 *Hex.*	4 *Hex.*
ADP						
μM	0·94	0·94	0·80	18·2	29·3	35·3
SA/μM ADP	3485	3320	3340	1460	1300	1255
ATP						
μM	0·83	0·78	0·66	27·9	12·1	2·4
SA/μM ATP	6430	6165	5605	3420	3070	—
G-6-P						
μM	0·02	0·02	0·02	0·01	17·0 (est.)	35·0 (est.)
SA/μM G-6-P	—	—	—	—	2300	2405

Data taken from Siekevitz and Potter (1955).

of an experiment in which some answer was attempted. We used a much larger amount of mitochondria in the oxidative phosphorylation medium so that we could measure the concentrations of ADP and ATP in the mitochondria as well as in the medium after spinning down the mitochondria. At the same time, by employing radioactive inorganic phosphate, we could obtain the specific radioactivities of these compounds and compare them with the radioactivity of the glucose-6-phosphate formed as a result of hexokinase activity. By choosing certain conditions we could also obtain different specific radioactivities for the ATP in the medium and the ATP in the mitochondria, so that we could determine the source of the phosphate of glucose-6-phosphate. It can be seen that the large amounts of added hexokinase make for a lower concentration of ATP in the medium but not for the ATP within the mitochondria. However, when we examine the specific activities of the glucose-6-phosphate, we can see that the phosphate of this compound could only have come from the terminal phosphate of the intramitochondrial ATP. We thus postulated that the added hexokinase was acting not on the ATP in the medium nor even on the ATP within the mitochondria but on the ATP as it was leaving the mitochondria, i.e. at the mitochondrial membrane. We then tried to verify the exactness of this site of action and Fig. 12 gives the results. We added a hexokinase solution of a known activity to mitochondria, and then, after spinning down the mitochondria, we tested the hexokinase activity of the supernatant solution remaining on top of the mitochondrial pellet. The difference between the two activities should be the amount of hexokinase which was brought down with (or bound to) the mitochondria. We thus expected 10 per cent of the activity to be brought down, but, as Fig. 12 shows, we obtained activities of from three to ten times this amount. In other words, we apparently increased our initial hexokinase activity by having it bound to mitochondria. Ernster and Lindberg (1952) have done much the same experiments and have obtained the same results. The possible reasons for this

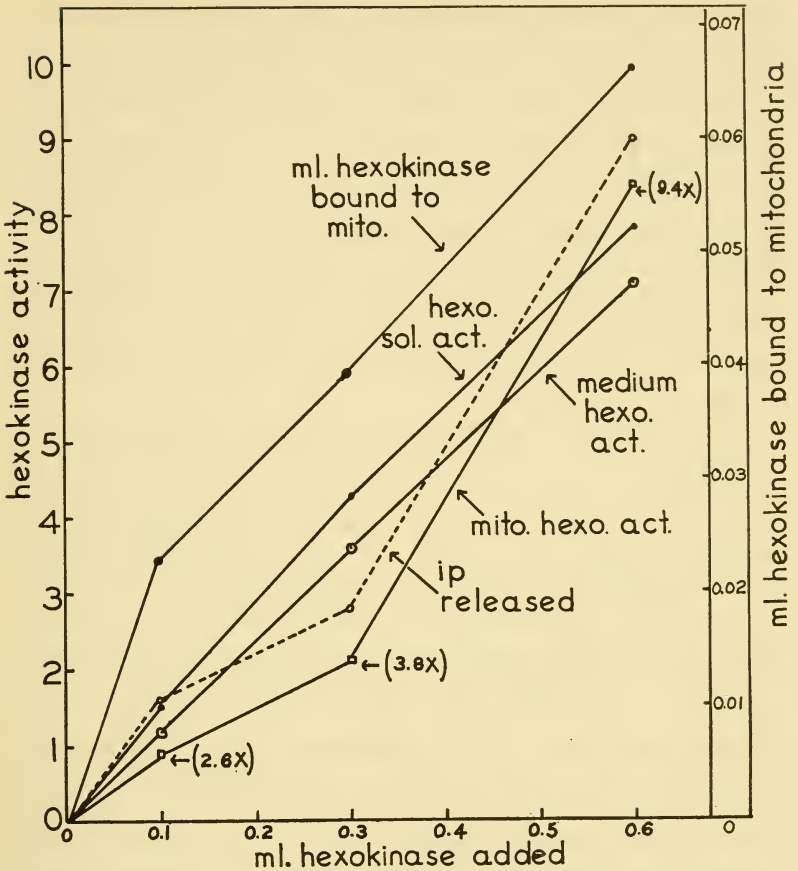

FIG. 12. Variations in hexokinase activity. The figure gives the variations in hexokinase activity as a function of the amount of hexokinase added to a mitochondrial suspension and then spinning the mitochondria down. Hexo. sol. act. = activity of original solution of hexokinase; medium hexo. act. and mito. hexo. act. = activity of the enzyme in the medium and in the mitochondrial pellet after the mitochondria were spun down; ml. hexokinase bound to mito. = calculated amount of hexokinase brought down with the mitochondria; ip released = amount of inorganic phosphate in the medium after the mitochondria were spun down and presumably due to the small amount of contaminated microsomal glucose-6-phosphatase activity in the large amount of mitochondria used. The figures in parentheses refer to the increase in hexokinase activity which was brought down with the mitochondria and it was calculated by comparing the obtained activity of the spun-down mitochondria with the difference between the activity of the original hexokinase solution and the activity of the hexokinase remaining in the medium after spinning down the mitochondria. Data taken from Siekevitz and Potter (1955) and unpublished experiments of Siekevitz and Potter.

apparently enhanced activity for the hexokinase bound to mitochondria are various: it could be due to an increase in the local concentration of the substrate, to a decrease in local concentration of the products, to differences in ion concentrations or pH between the surface of the mitochondria and the medium (cf. Danielli, 1944), or to an increased possibility of binding substrate to an orientated enzyme molecule. This latter reason could explain the findings (Wenner, Dunn and Weinhouse, 1953) that glucose oxidation by kidney, liver, and heart homogenates does not require the addition of ATP, for the concentration of ATP at the membrane site of action, while low, could still be sufficient to saturate the hexokinase at this site.

The possibility of enhancement of enzyme activity upon the orientation of the enzyme molecule onto a particular surface, has, of course, been raised by other authors, but the foregoing experiment is among the first to indicate that such a phenomenon can take place. The particular relevance of these observations to the cytological organization of enzymes lies in the further possibilities that enzymes which can be localized in the soluble matrix of the cell may not necessarily effect their activity there, but become "activated", you might say, only when they become bound to some intracellular membrane. This postulated movement of enzyme molecules onto and off membranes might well be the activity which is involved in the regulation of the course of substrate metabolism. Since hexokinase is one of the key enzymes involved in carbohydrate metabolism, we (Siekevitz and Potter, 1955) thought to see if a hormone, in this case insulin, had any effect on the binding of hexokinase to the mitochondria. Much to our disappointment, it had no effect, but it still is a good idea, to be investigated whenever a suitable test system becomes available.

Cori (1955) said that ultimately biochemistry must deal with integrated enzymic activity at the cellular level of organization rather than with individual enzyme reactions. He made this remark at the end of a paper dealing with some

aspects of carbohydrate metabolism. This particular topic makes a good subject for cytochemical speculation because, of all the metabolic processes, most is known about the reactions involved in carbohydrate metabolism. Let us now note what is known about the localization of the enzymes involved, particularly those catalysing the steps between glycogen and triose phosphate. Of these, phosphorylase and phosphoglucomutase (cf. de Duve and Berthet, 1954), glucose-6-phosphate dehydrogenase (cf. de Duve and Berthet, 1954; Newburgh and Cheldelin, 1956) have been found in the

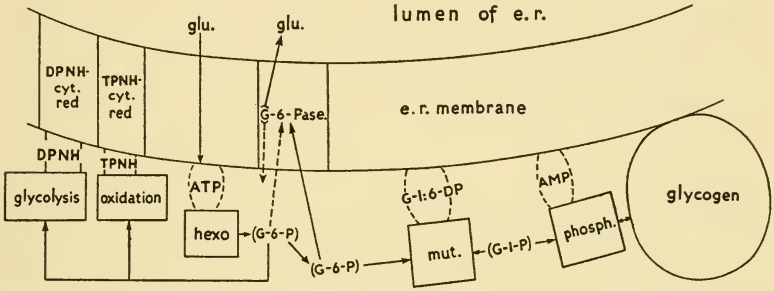

FIG. 13. Schematic representation of the possible rôle of the ER in some aspects of carbohydrate metabolism.

supernatant fluid along with aldolase. Because glycolysis can take place in the supernatant fluid, all the glycolytic enzymes are presumed to occur in this fraction (cf. de Duve and Berthet, 1954). Glucose-6-phosphatase is one of the few enzymes which has been exclusively localized in the microsome fraction (cf. de Duve and Berthet, 1954). The localization of hexokinase is controversial (cf. Siekevitz and Potter, 1955). As mentioned above, two auxiliary enzymes, the DPNH and the TPNH-cytochrome *c* reductases, are found in the microsomes as well as in the mitochondria.

Fig. 13 is an entirely theoretical scheme, based on very few observations, but including those mentioned above. I am purposefully exaggerating the morphological component of cellular metabolism in the hope that while I will undoubtedly

overshoot my mark, this will not happen in every case. I do not know whether glucose gets into the cell as I have depicted, by a pinocytic mechanism (Fig. 7) via the ER, as Holter and his co-workers have indicated happens with amoeba. Mr. Donald Young, in our laboratory, who has been working on the whole problem of glucose exit and entry into the liver cell as related to cell structure, has tried to show, by injecting radioactive glucose and separating microsomes, that this might also be the case for the liver, but without success. Since there are many hazardous chances to be taken in this sort of an experiment, we are not discouraged. However, it is suggested that some, if not all, of the glucose gets into the cell by passing into the ER after prior pinocytic entry, and then passing through the ER membrane to be immediately phosphorylated by ATP and hexokinase on the inner, cytoplasmic matrix, side of this membrane. The exit of glucose is visualized as being accomplished by the exclusively microsomal enzyme, glucose-6-phosphatase, and thus the function of this enzyme is thought to be that of secreting glucose into the vesicles of the ER, and then out of the cell. Both of these schemes take cognizance of the thought that glucose cannot get in and out of the cytoplasmic matrix without coming into contact with a transforming enzyme, hexokinase in the one case and glucose-6-phosphatase in the other. Thus, a barrier exists to the free interchange of intracellular and extracellular glucose, considering the glucose in the ER lumina as extracellular. This idea of the active transport of glucose through cell membranes by the action of a phosphatase is not new (cf. Danielli, 1952) and, in fact, has been successfully tested in the yeast cell by Rothstein and his co-workers (Rothstein and Meier, 1948; Rothstein, Meier, and Scharff, 1953) who found that hexose phosphatase, as well as adenosine triphosphatase (ATPase), are localized in the cell membrane, and by Sacks (1944, 1945), who has postulated as a result of his experiments that glucose-6-phosphate is formed in the muscle cell membrane, and is split there, with glucose going into the cell and phosphate remaining on the outside. What I further propose

is that a barrier to glucose penetration exists deep within the cell itself, and that the purpose of the glucose-6-phosphatase localized in the ER may be not only to secrete glucose to the exterior but also to remove glucose from contact with the glycolytic enzymes in the cytoplasmic matrix, thus in effect regulating, possibly one way among many, the pathways of glucose metabolism in the cell. As a corollary, it is suggested that the nucleus can grab its glucose out of the hungry mouth of the glycolytic enzymes in the cytoplasmic matrix by virtue of its sitting enclosed by the membranes of the ER (Fig. 7). I am in the dark as to whether a scheme of this sort can work in the manner pictured by Mitchell (1957), in which carrier compounds, which can be group-transferring enzymes, are located in the membrane and are thought to move compounds through by their own actual movements within the membrane.

The possible existence of a barrier within the cell has already been mentioned; some recent results have dramatically indicated the actuality (Lajtha, Berl, and Waelsch, 1959). Previously Waelsch (private communication) had found that the synthesis of glutamine from glutamic acid takes place in the liver and brain but not in the blood and, in the liver and brain, the microsome fraction is the most active in this synthesis. The surprising finding, related in Table VI, is that if radioactive glutamic acid is injected into the blood, and the liver and plasma quickly separated, the specific activity of the glutamine isolated from the plasma is higher in more than half the cases than is the specific activity of the glutamine in the liver. The same results appear even when non-radioactive glutamine is injected with the radioactive glutamic acid. The authors' tentative explanation is that the glutamic acid gets into the liver cell, is converted to glutamine in some compartment, and the glutamine gets out again without mixing with the rest of the glutamine in the liver cell. Since synthesis takes place mostly in the microsome fraction, they visualized the ER to be the site of this synthesis and to be the barrier between plasma glutamine and liver glutamine. There

Table VI

SPECIFIC ACTIVITIES OF GLUTAMIC ACID AND GLUTAMINE AFTER INJECTION INTO MICE

Expt.	*Comp. injected*	*Duration of Expt.*	*Organ*	*Sp. act. of isolated comp.*	
				Glutamic acid	*Glutamine*
1	^{14}C-glutamic acid	2′	plasma	45,000	5,200
			liver	2,800	2,100
2	^{14}C-glutamic acid	3′	plasma	8,300	4,500
			liver	12,000	20,000
3	^{14}C-glutamic acid	5′	plasma	6,000	1,900
			liver	1,200	850
4	^{14}C-glutamic acid	5′	plasma	22,000	4,100
			liver	2,400	2,000
5	^{14}C-glutamic acid	10′	plasma	8,000	3,000
			liver	9,200	21,000
6	^{14}C-glutamic acid and ^{12}C-glutamine	3′	plasma	130,000	700
			liver	1,600	2,000
7	^{14}C-glutamic acid and ^{12}C-glutamine	5′	plasma	20,000	12,000
			liver	3,200	3,500
8	^{14}C glutamic acid and ^{12}C-glutamine	5′	plasma	8,300	11,000
			liver	3,000	5,900

Data taken from Lajtha, Berl and Waelsch (1959).

can be other explanations, of course, but there is no evidence as yet which goes against the conclusions of these authors. It would, therefore, be intriguing and fairly easy to see if the glucose-6-phosphate to glucose system behaves in the same manner, and this is being done at present.

The rest of the scheme in Fig. 13 shows the possibility that the whole chain of events in the initial stages of glucose metabolism might take place at the surface of the ER membranes. For the sake of simplicity, several enzymes, including amylo-1 : 6-glucosidase, have been omitted. It has been suggested quite a few times in the past that the purpose of the DPNH- and TPNH-cytochrome *c* reductases in the microsome might be to act as coenzymes for glycolysis and in the initial

step of glucose oxidation. We know at present that probably both of these enzymes would seem to be a part of the membranes of the ER.

Now nothing is known concerning the structural relationships of glycogen in the cell; all that is known is that it is deposited in discrete amorphous masses in the cytoplasm. It may be assumed that both glycogen synthesis and breakdown take place at the cytoplasmic matrix surface of the ER membranes, via enzymes which react at those surfaces. In this view, glucose secretion by the liver is analogous to protein enzyme secretion by the pancreas, and perhaps to blood protein secretion by the liver, except that while the large protein molecules are secreted as such, by being packaged, the large glycogen aggregates are broken down to smaller molecules beforehand.

As regards the enzymes involved in the scheme, it is true that only glucose-6-phosphatase and the electron-transferring enzymes are situated in the microsome fraction. However, it is possible that the effective action of the other enzymes takes place at the cytoplasmic matrix surface of the ER, the hexokinase, for example, being "activated" at the ER membrane surface in the same way that it was "activated" at the mitochondrial surface. The additional compounds necessary for enzyme activity, ATP in the case of hexokinase, and the cofactors glucose-1 : 6-diphosphate and adenosine monophosphate (AMP) for phosphoglucomutase and phosphorylase, are depicted as possibly binding the enzymes to the ER membranes. In the first two cases the coenzymes act as phosphate donors to the substrate, while in all three cases the extra phosphate groups are thought to act by binding, not substrate to enzyme, but enzyme to membrane.

Now what are the biochemical means by which some of these enzymic reactions can be regulated? Firstly, according to the scheme, the concentration of glucose in the ER lumina is in equilibrium with the concentration of glucose in the blood, and thus the blood level could directly influence the glycogen-glucose equilibrium. In fact, Freedland and Harper (1957)

have found a correlation between increased gluconeogenesis and increased glucose-6-phosphatase activity when the supply of available glucose in the diet is reduced. In the fasted animals, glucose-6-phosphatase activity is also increased (cf. Weber and Cantero, 1957). It is my contention that the reduced glucose in the diet could lead to a temporary reduction of glucose in the blood, then in the ER lumina, and in some way to an increased phosphatase activity, leading, because of shifts in equilibria, to an increase in the breakdown of liver glycogen, and to a steady-state level of glucose in the blood. It is well known that the equilibrium of the concentrations of small molecules between liver and the blood is very rapid. Again, it has been suggested (Cahill *et al.*, 1958) that the rate of reoxidation of TPNH may control glucose-6-phosphate dehydrogenase activity, and this could be regulated, according to the scheme, by the rate of oxidation of this compound by the microsomal enzyme, the TPNH-cytochrome *c* reductase.

The rates of enzymic activity, in this case hexokinase, phosphoglucomutase, and phosphorylase, might be enhanced by being attached to the ER membranes. This could be accomplished in two ways; in the first, hexokinase could be "activated" according to the several biochemical considerations cited above; in the second, an inactive enzyme could be activated by a process taking place at the membrane surface. A theoretical example of the latter is phosphorylase, for an "inactive" phosphorylase *b* has to be converted to an "active" phosphorylase *a* (Cori, 1955; Cowgill and Cori, 1955) before catalysis can take place. It is known now that an enzyme, phosphorylase *b* kinase, does this converting by phosphorylating the enzyme in the presence of ATP (Krebs and Fischer, 1956; Rall, Sutherland and Wosilait, 1956). Is it not possible that this kinase is part of the ER membrane, and the process of activating phosphorylase in the cell consists of moving it from the cytoplasmic matrix to the ER membrane site?

It is not difficult to conceive how enzymes which are normally in a "soluble" state in the cytoplasmic matrix

become bound to cell structural elements. It is a different matter when we think of how this binding can be regulated. Piling hypothesis upon hypothesis, I would suggest that the hormones somehow control this movement of enzymes within the cell. Thus, the hormones involved in carbohydrate metabolism are depicted as not acting on the enzyme *per se* but in bringing enzyme, substrate, and cofactors together at a suitable surface, and they do this by the actual moving of the enzyme, complexing it perhaps, to the site where the substrate and cofactors are at the membrane surface. Even though we could not show it with the binding of hexokinase to mitochondria, it is still possible that insulin could act in such a manner to bind hexokinase to the ER membranes and thus increase its activity. In the present fragmented state of our knowledge one can only talk in general terms, but it is hoped that this discussion will give rise to specific experiments and to more definite conclusions.

I would like to end this presentation by citing some recent work which I think signifies more than we can guess at present; for I can give you a rousing climax to this sort of paper only by ending with a question mark. The ribonucleoprotein particles which can be detached from the microsomes of the pancreas still contain the enzymes which we think are synthesized at this site, such as amylase, ribonuclease (RNAase), chymotrypsinogen and trypsinogen (Siekevitz and Palade, 1958*b* and *c*). These enzymes are firmly attached to these particles, for they cannot be washed out with water or even with low concentration of DOC. We have no conclusive evidence at present that these enzymes are not non-specifically adsorbed onto the particles, but we are assuming for the present that they are specifically bound to these synthetic sites. However, as Table VII shows, these enzymes—and RNAase and chymotrypsinogen have been tested as well as amylase—do become detached if the particles are treated with 5×10^{-4} M-ATP or inorganic pyrophosphate. Inorganic phosphate and AMP will do the same but only at ten to twenty times higher concentrations. Mg^{2+} at low concentrations

Table VII

RELEASE OF AMYLASE ACTIVITY FROM MICROSOMAL NUCLEOPROTEIN PARTICLES

The particles were detached from microsomes from 200 mg. guinea pig pancreas. Incubated in 0·44 M sucrose with additions (total volume: 2·0 ml.) for 30 min. at 35°C; 8·0 ml. water then added and spun at 105,000 *g* for 90 min. Amylase activity tested in resultant pellet and medium.

	Per cent amylase activity	
Additions	*In medium*	*In pellet*
None	0	100
1 μM ATP	100	0
2 μM $MgCl_2$	0	100
1 μM ATP and 2 μM $MgCl_2$	0	100
1 μM ATP, 2 μM $MgCl_2$, 10^{-4} M DNP	54	46
1 μM ATP (0°C)	31	69
1 μM Pyrophosphate	100	0
1 μM Pyrophosphate, 2 μM $MgCl_2$	47	53

Data taken from Siekevitz and Palade (unpublished).

does not do anything at all but when we combine Mg^{2+} with ATP we obtained an inhibition of the releasing effect of ATP alone. It appears that the added ATP or pyrophosphate is complexing the Mg^{2+} in the particles, and this Mg^{2+} is necessary in keeping the enzymes onto the particles. But this is not the entire story. As Table VIII indicates, the particles treated with ATP or with pyrophosphate lose most of their RNA and only little of their protein. The RNA goes

Table VIII

RELEASE OF RNA FROM MICROSOMAL NUCLEOPROTEIN PARTICLES

The particles were detached from the microsomes from 1 g. guinea pig pancreas. Incubated in water or in 4 μM ATP/2·0 ml. for 30 min. at 35°C; 8·0 ml. water was then added, and spun at 105,000 *g* for 90 min. RNA and protein were determined in resultant pellet and medium.

	μg. RNA		*μg. Protein*	
Treatment	*In pellet*	*In medium*	*In pellet*	*In medium*
None	384	10	757	91
ATP	74	15	666	176

Data taken from Siekevitz and Palade (unpublished).

into the medium and, we think, begins to be attacked there by the RNAase which is simultaneously released from the particles. So now it would seem that the addition of ATP or pyrophosphate, or at higher concentrations AMP or phosphate, complexes the Mg^{2+} which binds the RNA to the protein of the particles. The enzymes are released from their possible site of synthesis by a process which includes the disruption of the synthesizing structure.

Does this happen *in vivo*? Is this the way these presumably newly-synthesized enzymes are released from their site of synthesis to their eventual destination, in this case the zymogen granules via the ER? Can we generalize and say that macro structures can be built or destroyed by such complexings which involve smaller molecules? DNP has an effect on this one particular complexing (Table VII); can other phenolic compounds have similar effect; can thyroxine? It is no startling revelation to proclaim that chemical mechanisms modify structural form; but we have now reached the brink where we can ask experimentally meaningful questions. Unlike a certain well-known statesman, let us not totter on this brink, for it is not one which borders on complete destruction; let us jump in, for the heady seas of the relationships of structure and function await us.

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DISCUSSION

Chance: I have a question on the function of the pyridine nucleotide cytochrome *c* reductase in the endoplasmic reticulum. Assuming that there is cytochrome *c* in the cytoplasm, what is the electron acceptor for cytochrome *c* reductase which would enable it to transfer electrons and thus to act as an ion pump mechanism? While there is surely cytochrome b_5 in the microsomal particle, there does not appear to be any oxidant for it. Therefore, while one could get a "substrate amount" of electron transfer, how can there be any continuous electron transfer in the endoplasmic reticulum? This point also refers to Palade's ideas about the endoplasmic reticulum of muscle. It is a question of whether this pyridine nucleotide cytochrome *c* reductase is in the endoplasmic reticulum of the muscle.

Siekevitz: No one knows about the electron acceptor for DPNH in the endoplasmic reticulum. We are working now on the question of the presence of a DPNH-cytochrome *c* reductase in muscle microsomes, and Dr. Luck in our laboratory has recently found this enzyme to be active in heart muscle microsomes.

Lynen: In the experiment where you added hexokinase to mitochondria and spun it down, you found that the sum of the activities of mitochondria and supernatant was much higher than the original hexokinase activity.

Siekevitz: Yes, and one of the causes might be dampened enzyme activity due to product inhibition. Inhibition of hexokinase by the product, glucose-6-phosphate, has been reported (Crane, R. K., and Sols, A. (1953). *J. biol. Chem.*, **203**, 273). When we had to use a large amount of mitochondria in order to get the mitochondrial nucleotides, we also brought down with it some of the microsomes which had glucose-6-phosphatase activity. We demonstrated that the mitochondrial preparation had some of this glucose-6-phosphatase activity; so the activation might be due to a relieving of the inhibition caused by glucose-6-phosphate.

Racker: Did you use yeast hexokinase in these experiments?

Siekevitz: Yes.

Racker: Yeast hexokinase is not very susceptible to product inhibition.

Siekevitz: We know that we obtained a breakdown of glucose-6-phosphate simultaneously with, and proportionally to, the increase in activity of the hexokinase which was bound to the mitochondria.

Lynen: How did you measure hexokinase activity?

Siekevitz: By the changes in the nucleotide concentrations.

Lipmann: Crane and I did some experiments where we added radioactive phosphate to a dense suspension of mitochondria, and it was found that in the very early part, within the first minutes, the specific activity of the ATP proved to be greater than that of inorganic phosphate. This was very paradoxical. We then found that this was caused by a dilution of the inorganic phosphate by intramitochondrial phosphate which had not yet equilibrated (Crane, R. K., and Lipmann, F. (1953). *J. biol. Chem.*, **201**, 245). I have some difficulty in understanding how such an observation compares with yours.

Siekevitz: Our explanation is based firstly on the finding that we can only get this effect by adding ADP, not AMP. The ADP would be reacted upon by myokinase to give ATP on the surface and AMP inside. The AMP on the inside would eventually go to $AT^{32}P$ so that this $AT^{32}P$ inside the mitochondria would be much higher in specific radioactivity than the mixture of $AT^{31}P$ and $AT^{32}P$ on the outside.

Lipmann: It seems, then, that the two sets of experiments cannot be directly compared since we used AMP, and we assayed early when the internal phosphate had very low specific activity, in other words, when it was very far from being in equilibrium with the external phosphate.

Siekevitz: Yes, we also noticed that the intramitochondrial inorganic phosphate is lower in specific radioactivity, but only by 30 per cent, than the extramitochondrial inorganic phosphate, and the inner phosphate has the same specific activity as the intramitochondrial terminal ADP phosphate. Therefore, the newly synthesized inner ATP must have come from the pool of mitochondrial inorganic phosphate; and the differences in specific activity between intra- and extramitochondrial ATP could not have been due to differences in inorganic phosphate radioactivity.

Bartley: To return to the structure of the nuclear membrane. J. D. Robertson (1959, *Biochem. Soc. Symp.*, in press) showed electron micrographs which indicated that the nuclear membrane was not attached to the endoplasmic reticulum. The perinuclear pool was connected with the exterior of the cell by the channels between the endoplasmic reticulum. This view fits in better with studies on isolated nuclei which apparently have their own bounding membrane. In Robertson's view the fluid bathing the nucleus is connected with the extracellular fluid. In your view the nucleus is in contact with the intracellular fluid.

Siekevitz: According to the theory held in our laboratory, the nucleus itself has no membrane. It is enclosed by the double membrane system of the endoplasmic reticulum. Perhaps, when you isolate the nucleus you also get part of this endoplasmic reticulum membrane.

Bartley: In electron microscope pictures of isolated nuclei there is no

sign of pieces of endoplasmic reticulum remaining attached to the nuclear membrane.

How do you reconcile the system of intracellular membranes with the mitochondrial movements that go on in the living cell? The membranes would seem to limit the regular movements that you get with mitochondria.

Siekevitz: If you have a system of channels formed by the reticulum membranes then the mitochondria may just move back and forth between two adjacent channels.

Bartley: Have you any idea as to what is the force causing the movement?

Siekevitz: Bennett (1956, *loc. cit.*) has published a theory that the whole membrane system is in motion through the cell. To go back to the nuclear picture, he thinks that if you have membranes of the endoplasmic reticulum enclosing the nucleus, the nucleoprotein particles which are on the endoplasmic reticulum membrane might come from the RNA elaborated by the nucleus and be deposited on the membranes lining the nucleus, and then by the actual movement of the membrane become the nucleoprotein particles of the endoplasmic reticulum (microsome) fraction. There is no evidence for this, but there is one piece of evidence against it, and that is that the RNA of these particles has a very low turnover as compared to the RNA you get from the nuclei.

Aldridge: On the hexokinase question, we found that with mitochondria we can stimulate fourfold by the addition of hexokinase, but if we titrate with minimal amounts of hexokinase up to maximum stimulation and also measure the inorganic phosphate uptake under those conditions, we find that the amount of hexokinase we add is not sufficient to account for the phosphate uptake; there is a threefold differentiation.

Dr. Siekevitz, you have described an experiment in which the nucleotide levels were measured upon adding hexokinase. I have recently carried out a similar experiment with the addition of DNP. The liver mitochondria used have excellent stability, for the addition of potato apyrase to them after they have been metabolizing pyruvate at 37° at the slow unstimulated rate for 2 hours still produces a fourfold stimulation.

Titration with DNP increases oxygen uptake until a plateau is reached at 1×10^{-5}M (4·1-fold stimulation). The concentration of ATP is maintained right up to maximal stimulation of oxygen uptake and only falls and ADP increases when more DNP is added (Fig. 1 p. 48). This probably has physiological significance and also explains why it is so difficult to detect a fall in ATP concentrations in the tissues of animals after non-lethal doses of DNP.

Siekevitz: That agrees with some results we had, with addition of DNP and hexokinase, on the ability of the mitochondria to maintain the ATP at maximum stimulation.

Potter: This is almost a perfect duplication of our data. Our interpretation was that here we were measuring the total nucleotides. We

went on to show that since what is in the mitochondria is so small in comparison with the total in the flask then this does not, in fact, measure the ratio of the nucleotides inside the mitochondria. The experiments that were done on the conversion of ornithine to citrulline permitted the conclusion that on the ascending limb of the oxygen uptake curve there was a lower ATP : ADP ratio within the mitochondria as deduced from the intramitochondrial conversion of ornithine to citrulline.

Chance: On the question of the relationship of the endoplasmic reticulum to the mitochondria and the existence of a cytoskeleton, Dr. Siekevitz, you have postulated some organization of the cytoplasm

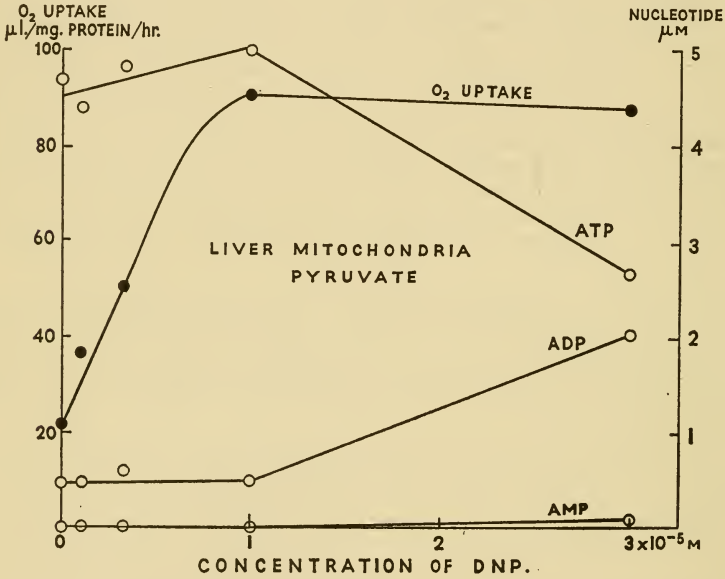

FIG. 1 (Aldridge). The effect of 2 : 4-dinitrophenol upon the oxygen uptake and nucleotide concentration when liver mitochondria are oxidizing pyruvate. Oxygen uptake was measured between 10 and 30 min. and nucleotide concentrations at 32 min. after addition to the bath at 37°.

with respect to the mitochondria. We agree with Bartley's comments (see p. 46, above), Also, Dr. Bo Thorell of the Pathology Department, Karolinska Institute, and I did the following simple experiment. In the ascites tumour cell there is a delicately balanced regulation initiated by adding glucose to the cells as taken from the mouse. This regulation apparently involves some intimate relationship of cytoplasmic and mitochondrial enzymes. We centrifuged the cells in gum arabic for 60 minutes at the top speed of the Spinco centrifuge, so as to separate

the nucleus, the mitochondria and the cytoplasmic elements in order to determine whether this would abolish or enhance metabolic regulation. We observed that the interior of the cell had been disrupted, i.e. the nucleus, mitochondria and fat bodies had moved to such an extent that we considered this as rather a dramatic effect. Then we took a portion of the cells to see whether the metabolic regulation caused by glucose addition would still function: it did. Thus, if there is a cytoskeleton which is relevant to the interactions of glycolysis and oxidation, it is one which has a considerable degree of mechanical stability.

Siekevitz: I can answer this by alluding to the old controversy of whether there is RNA in the mitochondria. If you isolate mitochondria, take a picture and develop it, you find a good many of these mitochondria still encapsulated within some endoplasmic reticulum membranes, a portion of which has come down with the mitochondria. It is very difficult to wash out these membranes; in fact, it just cannot be done. We are left with the controversy and with the problem of the degree of stability, contactness or "togetherness", which exists between mitochondria and microsomes.

Chance: I think there is a lot to be said in your favour, from the standpoint of the permeability of isolated mitochondria to added pyridine nucleotide. They are apparently impermeable. Is that due to the fact that they are prepared in "sealed packages" enveloped in the endoplasmic reticulum? I do not believe that anybody has proved this but it should be considered here.

King: Dr. Siekevitz, in your Table I the ratios

$$\frac{\text{specific activity of mitochondria}}{\text{specific activity of mitochondrial membrane}}$$

are about the same for succinoxidase and cytochrome oxidase, but quite different for DPNH cytochrome *c* reductase. I wonder why. Secondly, how have you tested succinoxidase, cytochrome oxidase and DPNH cytochrome *c* reductase?

Siekevitz: We tested by the usual procedure; succinoxidase with added cytochrome *c* and also in the presence of calcium and aluminum salts; cytochrome oxidase by the oxidation of reduced cytochrome *c*; and DPNH cytochrome *c* reductase by DPNH oxidation, i.e. by the usual spectrophotometric or manometric means. We break up the mitochondria with detergent, which is a fairly easy process. All we can say is that perhaps the DPNH cytochrome *c* reductase was once on the membrane, but it is much more easily broken off than is the succinoxidase or the cytochrome oxidase. We have no other explanation of why that enzyme was in the supernatant from the broken up mitochondria, while the other enzymes could be sedimented with the membranes.

SOME TOPOGRAPHICAL ASPECTS OF THE REGULATION OF CELL METABOLISM

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LIFE has been defined by Hopkins as "a dynamic equilibrium in a polyphasic system". For many years, the efforts of biochemists have been mostly directed toward a proper elucidation of the first half of this definition. They have indeed been uncommonly successful and expressions such as steady state, limiting step, key reaction, alternative pathways, competition, coupling, feedback, have become standard parts of a new jargon of biochemical cybernetics.

With the advent of electron microscopy and of differential centrifugation, it has become possible to explore also the second part of Hopkins' definition, which corresponds to the structural factors of cellular organization. These must be considered as bringing additional levels of complexity and new sets of relationships into systems which are already strongly organized by virtue of the multiple dynamic relationships existing between their various components.

One of the simplest consequences of structural organization is the fact that given groups of enzymes are associated together and separated from others. That most enzymes are indeed segregated in this manner has been abundantly verified in recent investigations, but the results obtained have not always been as clearcut as our desire for simplicity and conformity would have them to be. As pointed out in previous reviews (de Duve and Berthet, 1954; de Duve, 1957), insufficient resolution of the separation methods and of the assay procedures, together with other technical difficulties and artifacts, are partly responsible for this state of affairs. There are, however, other causes, amongst which our ignorance and

preconceived ideas of cellular organization play a not insignificant part.

A direct consequence of the segregation of enzymes is that media of different composition tend to form on each side of the membranes separating distinct enzyme systems. Thus, enzymic heterogeneity itself becomes responsible for the unequal distribution of numerous other cell constituents and for the appearance of diffusion gradients, membrane potentials and other manifestations of heterogeneity at the various interfaces. The exchanges governed by these phenomena may be of paramount importance in the regulation of cell metabolism.

A higher level of organization is reached if the catalysts which are part of a given unit are themselves associated structurally in a manner which either increases their efficiency as a system or provides the system with special properties. That such organized chains of enzymes may be present in the insoluble framework of the cell now appears fairly probable and it has even been maintained that soluble catalysts may be grouped in labile polyenzymic functional units in the intact cell.

Finally, one should not forget that the structural components of cells are themselves in a dynamic state. We are at present almost entirely ignorant of the mechanisms whereby intracellular units such as mitochondria are renewed or multiplied. This is obviously a problem of primary importance.

Investigations from our laboratory on the intracellular location of hydrolases may serve to illustrate the main physiological aspects of enzyme segregation. This work, which has recently been reviewed in detail (de Duve, 1959), has led to the identification in liver and several other tissues of a distinct group of cytoplasmic particles, termed "lysosomes" and containing a number of soluble acid hydrolases with an acid pH optimum. These enzymes include a phosphatase, a ribonuclease, a deoxyribonuclease, a cathepsin, a β-glucuronidase and an aryl-sulphatase and, therefore, have a combined

spectrum of activity which encompasses many important cell constituents. They are retained within the lysosomes by a membrane which effectively prevents their access to external substrates, and they are released in soluble and fully active form by all treatments which damage this membrane. Present indications are that lysosomes may represent only a very small fraction of the cell content and correspond with some type of dense bodies occasionally seen in tissue sections.

The most obvious implication of these findings is a negative one. They show that the soluble acid hydrolases are separated from most other cell constituents by a barrier which prevents them from acting on those constituents. In the present case, this negative aspect of segregation appears to be particularly important, since it concerns a group of enzymes which might very well cause considerable damage and even death if they were free to act within the whole cell. We have, in fact, been able to demonstrate that lysosomes release their enzymes very rapidly in necrotizing tissue.

The positive aspect of segregation is provided by the nature of the enzymes which are found to be associated and to form, therefore, what may be assumed to be a true physiological system. Association is more difficult to demonstrate unequivocally than separation, and we are not entirely certain that the lysosomal enzymes are all grouped together within a single type of particle. However, the bulk of the evidence is compatible with such a possibility and authorizes some speculation on the significance of the suspected association. This has led to the consideration that the only process in which the lysosomal enzymes could possibly be involved *jointly* is one of digestion of objects of complex chemical composition. Since liver lysosomes do not appear in bile and since similar particles exist in tissues other than digestive glands, the digestive phenomena with which they are concerned are taken to be intracellular and linked with engulfing processes such as phagocytosis, athrocytosis and pinocytosis. Some measure of support for this hypothesis has been furnished by the work of Straus (1956, 1957) on kidney lysosomes,

but the exact relationship between these particles and engulfing vacuoles remains to be established.

The example just mentioned is a special one in that the observed association could not be foreseen from previous biochemical data and represents, in fact, the only reason for considering the enzymes studied as linked together functionally. This is not the case for enzymes catalysing sequential reactions and associated in one of the accepted metabolic schemes of dynamic biochemistry. The existence of such schemes may, however, be misleading, since it is sometimes forgotten that they rest not only on facts but also on our interpretation of these facts and even on such chance factors as have led to the elucidation of a given sequence of reactions before another. In this respect, enzyme distribution studies may provide information of paramount importance and help us to distinguish between physiological and artificial polyenzymic systems.

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DISCUSSION

Lehninger: Prof. de Duve, have you had occasion to repeat the experiments of Dr. Straus (1957, *loc. cit.*) using your own method of collection to see whether the peroxidase which Straus used is actually liberated in the course of the lysis of the lysosomes? In his experiment a small amount of crystalline peroxidase from horse radish was injected intravenously into rats, and after a short time he isolated the lysosome fraction and demonstrated the presence of the peroxidase which was originally introduced intravenously in the droplets. This is a very important piece of evidence for the pinocytosis theory.

de Duve: No, we have not done so because Dr. Straus is shortly joining us for some time, and we hope to be able to do this work with him.

Greville: To refer to the matter of opening up enzymes in the mitochondria, unmasking or rendering them less occult. Dr. J. B. Chappell and I (1958, *IV int. Congr. Biochem.*, Abstr. of Communications, p. 73)

have carried out experiments with the enzyme, rhodanese, similar to those of Prof. de Duve and his colleagues. After consultation with Prof. de Duve and Dr. Bendall we carried out an experiment very similar to one which Prof. de Duve has described, the effect of hypotonicity—15 minutes at 0° with various concentrations of sucrose—on the activity of mitochondrial rhodanese. The curve we got is almost identical with one obtained by Prof. de Duve. Obviously, then, the rhodanese in mitochondria behaves in that respect similarly to the glutamic and malic dehydrogenases. An interesting point is that all measures which make the mitochondria swell seem to make the rhodanese active, e.g. addition of thyroxine, phosphate, oxidizable substrates, etc. We have tried the action of various combinations of these agents on swelling and we can match the swelling effects with the effects on rhodanese.

de Duve: I am very interested in this work of Drs. Greville and Chappell. It is particularly interesting because the substrates of rhodanese are so small. Here, we are dealing with very small ions, and if the swelling effect causes activation it could perhaps be due to the fact that these ions can penetrate swollen mitochondria but not shrunken ones.

In our laboratory, Dr. Bendall has tried to verify whether there is a state of osmotic swelling of the mitochondria where the membrane has become permeable to the substrates of glutamic dehydrogenase, without however allowing the enzyme to leach out, and from which reversal to the original lack of permeability is still possible in isotonic medium. He has obtained some indications that this might occur but his results were not conclusive.

Siekevitz: For purposes of metabolic control, do you think from your experiments that since each of the enzymes comes out at a slightly different peak, that might be an indication that one enzyme is in one particle and another one in another particle? Is there any way of separating them?

de Duve: We can separate crude subfractions in which the ratios of two enzymic activities differ to some extent, but the meaning of this is not clear. To verify your hypothesis, one would have either to effect a fairly clearcut separation of the two activities or to measure the enzymic content of single particles. We are a long way from there.

Estabrook: Have you studied the distribution of DPNH cytochrome *c* reductase in the same manner as you did cytochrome oxidase? The question comes from Dr. Siekevitz's paper as to whether the b_5 enzyme of the DPNH cytochrome *c* reductase which is antimycin-insensitive is always associated with mitochondria. Your distribution studies would perhaps answer that.

de Duve: According to the investigations performed in our laboratory by Dr. Pressman, liver microsomes contain a cytochrome *c* reductase which is entirely insensitive to antimycin and accounts for approximately 70 per cent of the total activity. The remainder is associated with the mitochondria in what appear to be two distinct forms. About one-half of the mitochondrial activity appears to be readily accessible to external substrate and is insensitive to antimycin; the other half,

which is completely inhibited by antimycin, reacts with external substrate only after the particles have been injured.

Chance: One can demonstrate spectroscopically, by adding DPNH to a suspension of intact mitochondria, the reduction of a pigment characteristic of the microsomal fraction. Whether this is microsomal contamination or whether there is naturally some of this pigment in the mitochondria, is not known. But if the endoplasmic reticulum is very close to the mitochondria, it would be very difficult to prove that no endoplasmic reticulum contaminates the mitochondria. The "opening" of the mitochondria would activate and provide a pathway from the cytochrome reductase to oxygen.

de Duve: This is a very interesting point, for if we calculate microsomal contamination on the basis of glucose-6-phosphatase activity we find that it is too low, by a factor of 4–5, to account for the antimycin-insensitive cytochrome *c* reductase activity of the mitochondria. If, therefore, the latter activity is due entirely to portions of the endoplasmic reticulum adhering to the mitochondria, then it follows that these portions are relatively richer in reductase and poorer in glucose-6-phosphatase than the bulk of the microsomes. In view of Dr. Siekevitz's remarks, this would tend to locate the microsomal reductase in the immediate neighbourhood of mitochondria within the cell.

Slater: I would like to have thought that it was as Prof. Chance said, because it is obviously simpler to have two enzymes doing the same thing instead of three. However, there is other evidence that this external cytochrome *c* reductase which is insensitive to antimycin, which was discovered by Dr. Lehninger, is not due to microsomal contamination, since you find exactly the same kind of behaviour with heart sarcosomes (Hülsmann, W. C., unpublished; Avi-Dor, Y., Traub, A., and Mager, J. (1958). *Biochim. biophys. Acta*, **30**, 164). There is very little evidence that any microsomes containing cytochrome b_5 are present in the heart.

Chance: There is more than one kind of cytochrome *c* reductase in the cytochrome b_5-containing microsomes. But one does not find cytochrome b_5-containing microsomes in the muscle even though one surely has an endoplasmic reticulum. What I wanted to know was whether someone has demonstrated a pyridine nucleotide cytochrome *c* reductase in endoplasmic reticulum of muscle. Dr. Slater, you say you have. Therefore, it is quite feasible that your phenomenon could have been attributed to a cytochrome *c* reductase of the endoplasmic reticulum of muscle cells which did not contain cytochrome b_5.

Slater: You now have three cytochrome reductases again, but you have two of them in the microsomes; i.e. you have two different cytochrome *c* reductases in the microsomes. The evidence I mentioned could only exclude the one cytochrome reductase which I know about. There is also no glucose-6-phosphatase in heart homogenate. We have tried it too and there is nothing corresponding to this liver microsomal fraction in heart homogenate.

de Duve: Glucose-6-phosphatase is only present in liver and in kidney.

Coxon: Prof. de Duve's reference to two deoxynucleases of different

pH optima calls to mind the old calculation of the number of hydrogen ions that would be found in a high-power microscope field (Peters, R. A. (1929). *J. State Med.*, **37**, 1), which would work out at about one per particle of the size we are considering. I wonder whether the mere switch of hydrogen ion concentration in a very small localized situation might sometimes affect the activation or otherwise of some of these enzymes.

de Duve: We can safely say that these are two distinct enzymes, when they are studied in disrupted systems. The alkaline one, which requires magnesium, is completely inhibited by ethylenediamine tetra-acetic acid, whereas the acid one is not affected by ethylenediamine tetra-acetic acid, and requires no magnesium. On the other hand, the acid DNAse is best studied at fairly high ionic strength, between 0·1 and 0·2, whereas the alkaline one is inhibited by high ionic strength.

Coxon: Would either of them be active in living cells?

de Duve: That is quite a different point, and we do not know that. They could be entirely inactive.

Estabrook: With reference to Prof. Slater's comment, Prof. Potter, you published that heart muscle homogenates were at least 90 per cent sensitive to antimycin. This was the difference between liver and heart muscle; the antimycin sensitivity of the DPN system.

Potter: In the liver homogenates there was complete sensitivity when no DPN was added. Insensitivity was observed on addition of external DPN.

Lehninger: In regard to lysosomes, in what different tissues have you found these particles, Prof. de Duve?

de Duve: The answer to this question depends on what type of evidence is needed before one is allowed to conclude that lysosomes are present in a tissue. In liver, for which we have the most complete case, the following criteria are fulfilled. (1) The acid hydrolases of the lysosomal group are present largely in particulate form and are therefore sedimentable in a properly prepared homogenate. (2) When present within intact particles, they are essentially unreactive towards external substrates. (3) They are released in soluble and fully active form by a variety of treatments which injure the particles. (4) By the use of special techniques, the particles which contain these hydrolases can be partly separated as a group both from true mitochondria and from microsomes. (5) All lysosomal hydrolases are liberated in the same proportion when the activating treatment is graded so as to cause only a partial release of the enzymes.

The next best case is on kidney, where Straus (1956, 1957, *loc. cit.*) has clearly shown criteria 1 and 4 to be fulfilled and has obtained some evidence, which we have confirmed, for criteria 2 and 3. Brain, which has been extensively studied in my laboratory by Miss Berleur, answers criteria 1, 2 and 3, but so far has failed to conform to criterion 4, presumably in view of the technical difficulties inherent in the fractionation of such highly heterogeneous material. Finally, indications in favour of criteria 1 and 2 have been obtained on spleen, thyroid and, according to the literature, pancreas and pituitary, as well as on amoebae. We are

still a long way from making lysosomes into an obligatory component of the "typical cell". But, at least, there is as yet no evidence to disprove this possibility. I should perhaps add that the capacity to segregate circulating proteins, which may be associated with lysosomes, has been found by Straus (personal communication) to be a property of most tissues.

Siekevitz: To comment on DPNH cytochrome *c* reductase: we have been doing experiments with liver microsomes and liver mitochondria containing these enzymes. We can solubilize the enzymes from the microsomes and the mitochondria in the same way with deoxycholate. As I have shown before, the microsomal enzyme can be resedimented if we dilute out the detergent, but the mitochondrial enzyme does not come down after this dilution. There is thus quite a different structural relationship involved between the DPNH reductases of the mitochondria and of the microsomes.

CONTROL OF RATE OF INTRACELLULAR RESPIRATION

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Aerobic cells are able to bring about the complete oxidation of glucose, according to reaction (i)

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O \qquad \text{(i)}$$

which is catalysed by a complex system of enzymes within the cell. If glucose and oxygen are introduced into the cells containing these enzymes, reaction (i) predicts that glucose will be oxidized at a rate governed by the concentration of the enzymes. However, it is well known that animals can vary the rate of their metabolism, and their oxygen consumption, according to their needs. For instance, the oxygen consumption of the working leg muscle of a man can be increased about sixty-five times during the performance of work (Asmussen, Christensen and Nielsen, 1939). The heart muscle, also, although normally it works at fairly constant activity can increase its oxygen consumption appreciably and, more interestingly, it is possible to stop the oxygen consumption of the heart by bringing about cardiac arrest by injection of potassium citrate into the coronary circulation (Melrose *et al.*, 1955).

There must, therefore, be mechanisms controlling the rate of reaction (i). One mechanism, which is under hormonal control, concerns the rate at which glucose can enter the cell. Our present discussion will not, however, deal with this question, but will be concerned with possible mechanisms which control the rate of oxidation of the intracellular glucose and other products of the digestion of foodstuffs.

A mechanism of control of the respiratory rate which is

probably important *in vivo* was uncovered by Lardy (1952). It has been known for more than twenty years that respiration is linked with the esterification of inorganic phosphate. Using mitochondria isolated from rat liver by a procedure designed to yield a preparation devoid of adenosine triphosphatase (ATPase) activity (Kielley and Kielley, 1951), Lardy and Wellman (1952) were able to show that the link between oxidation and phosphorylation was compulsory, i.e. respiration did not occur in the absence of the components necessary for oxidative phosphorylation.

Reaction (i) must, then, be extended to include the phosphorylation reaction, thus:

$$C_6H_{12}O_6 + 6\ O_2 + 38\ ADP + 38\ H_3PO_4 \rightarrow 6\ CO_2 + 44\ H_2O + 38\ ATP \quad \text{(ii)}$$

Since cells contain only a small amount of adenosine diphosphate (ADP), respiration will stop as soon as it is all phosphorylated to adenosine triphosphate (ATP). When the cell is stimulated to do work, e.g. in muscular contraction, the ATP is broken down

$$38\ ATP + 38\ H_2O \rightarrow 38\ ADP + 38\ H_3PO_4 + \text{work} \quad \text{(iii)}$$

The ADP liberated in this reaction now supplies the missing component on the left-hand side of equation (ii), so that respiration will commence, and the ATP is resynthesized. A kinetic steady state is soon set up, in which the concentration of ADP, and therefore the rate of respiration, is governed by the relative activities of the enzyme systems bringing about reactions (ii) and (iii).

Stimulation of respiration by ADP

Experiments illustrating the effect of the addition of ADP on the rate of respiration of rat liver mitochondria and guinea-pig heart mitochondria (sarcosomes) are shown in Figs. 1 and 2, respectively. In both cases, there is a sharp increase in the rate of oxygen uptake on the addition of ADP,

and a sharp break in the rate when all the ADP is phosphorylated. These experiments were carried out manometrically with reaction mixtures containing Mg^{2+}. The rapidity with which the new rate of respiration sets in after the addition of ADP and the sharpness of the "cut-off" when all

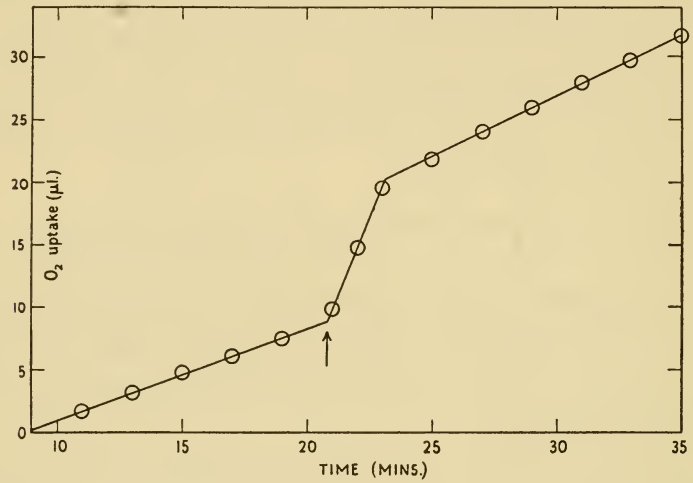

Fig. 1. Respiratory control in rat liver mitochondria. Reaction mixture: phosphate, pH 7·4, 0·016 M; NaF, 0·012 M; $MgCl_2$, 0·006 M; KCl, 0·083 M; ATP, 10^{-4} M; ethylenediaminetetra-acetate, 0·001 M; glutamate (DL), 0·007 M; sarcosomes, 0·6 mg. protein/ml. Reaction vol., 1 ml.; temp. 25°. Phosphate acceptor (a mixture of AMP and ADP) was added at the arrow. The measurement of O_2 uptake commenced 8 min. after the beginning of the experiment. The P/O ratio, calculated from the amount of O_2 required to phosphorylate the amount of phosphate acceptor added, was 2·60.

the ADP is phosphorylated are shown more clearly in the experiments of Chance and Williams (1955), who followed the change of oxygen concentration with an oxygen electrode.

The requirement of ADP and inorganic phosphate for intracellular respiration can be accounted for by the following formulation of oxidative phosphorylation, which is an elaboration of that suggested earlier (Slater, 1953). Indeed, the elaborations are unnecessary to explain the effect of ADP

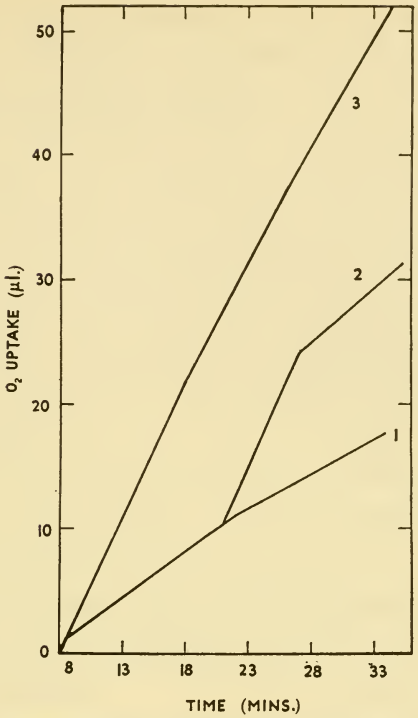

FIG. 2. Respiratory control in guinea-pig heart sarcosomes, isolated in 0·23 M sucrose, 0·005 M ATP, pH 7·4, and suspended in 0·25 M sucrose, 0·001 M ATP, pH 7·4. Reaction mixture as in Fig. 1. Curve 1, no further addition. Curve 2, ADP (2·7 μmoles) added at 19 min. Curve 3, 0·02 M glucose + hexokinase present from beginning. The measurement of O_2 uptake commenced 8 min. after the beginning of the experiment.

and inorganic phosphate, but have been made to account for other findings (Myers and Slater, 1957*b*; Slater, 1955, 1958; Purvis and Slater, 1959).

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2 \quad (1)$$

$$A \sim I + X \rightleftharpoons X \sim I + A \quad (2)$$

$$X \sim I + P_i \rightleftharpoons X \sim P + I \qquad (3a)$$

$$X \sim P + ADP \rightleftharpoons X + ATP \qquad (3b)$$

(P_i is inorganic phosphate. X and I could be interchanged in the last two reactions, i.e. the intermediate might be $I \sim P$, rather than $X \sim P$).

AH_2 and B are adjacent members of a phosphorylative step in the respiratory chain, e.g. AH_2 could be reduced diphosphopyridine nucleotide (DPNH) and B could be flavoprotein (fp.) I is a postulated intermediate which enables the energy made available in the oxido-reduction between AH_2 and B to be retained in the energy-rich compound $A \sim I$. This energy is then transferred to a second hypothetical compound X in reaction (2), giving $X \sim I$, and restoring A, the member of the respiratory chain. A is then reduced to AH_2 in the preceding step of the respiratory chain, and BH_2 is oxidized to B in the following step. In reactions (3*a*) and (3*b*), the energy of $X \sim I$ is used to make ATP, and free X and I are liberated. It is believed that there are three different I's (I_1, I_2 and I_3) corresponding to the three phosphorylative steps in the chain (Hülsmann and Slater, 1957). The question of whether the same or different X's are involved in the different steps is left open.

According to this scheme, the continuity of respiration requires that X and I are reformed from $X \sim I$. This is brought about by reactions (3*a*) and (3*b*) which require inorganic phosphate and ADP, respectively. Thus respiration proceeds, with the synthesis of ATP, until either the inorganic phosphate or the ADP is exhausted. This provides a control mechanism whereby the rate of synthesis of ATP is governed by the needs of the cell for ATP.

Stimulation of respiration by uncoupling agents

The extent to which the respiratory rate is under the control of the ADP concentration depends upon the stability, in the absence of ADP, of the various energy-rich intermediates in the above scheme, namely, $A \sim I$, $X \sim I$ and $X \sim P$. If

any of these intermediates can react with water, respiration will proceed even in the absence of ADP, and without the concomitant synthesis of ATP, i.e. the phosphorylation is "uncoupled" from the respiration.

Fig. 2 shows that isolated heart sarcosomes, in the presence of added Mg^{2+}, are appreciably "uncoupled", since they respire even in the absence of ADP. This "uncoupling" is not observed in short-time experiments in the absence of added Mg^{2+} (Chance and Baltscheffsky, 1958; Packer, 1957). The possible nature of this uncoupling will be discussed later. First, it is necessary to discuss the ways in which it is possible to uncouple freshly prepared liver mitochondria, which have very little respiration in the absence of ADP even in the presence of Mg^{2+}.

Uncoupling can be brought about in many ways, e.g.

(i) by the addition of 2 : 4-dinitrophenol (DNP), or other uncoupling agents;
(ii) by ageing of the mitochondria;
(iii) by the addition of a preparation which can be isolated from aged mitochondria.

2:4-Dinitrophenol

The stimulation of respiration of rat heart sarcosomes caused by the addition of DNP is shown in Fig. 3. The initial rate of respiration is about the same as that in the presence of ADP. For the following reasons, it is believed that DNP promotes the hydrolysis of $X \sim I$, rather than of $A \sim I$ or $X \sim P$:

(i) The DNP-stimulated ATPase reaction of mitochondria is not affected by the rate of oxidation or reduction of members of the respiratory chain (Myers and Slater, 1957*b*). Thus, A cannot be involved in the ATPase reaction.
(ii) The addition of DNP abolishes the requirement of inorganic phosphate for respiration (Loomis and Lipmann, 1949). Thus, the hydrolytic reaction must

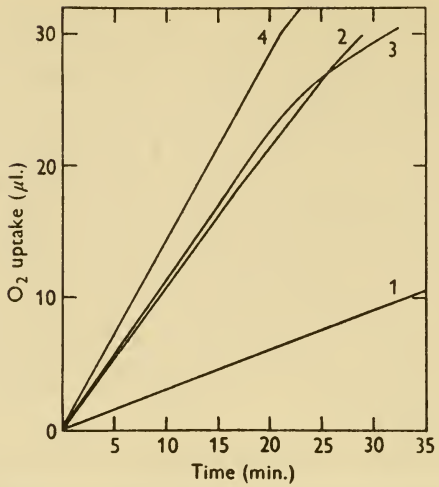

Fig. 3. Respiratory control with rat heart sarcosomes, isolated as in Fig. 2 and studied in the same reaction mixture. Curve 1, no further addition. Curve 2, 0·02 M glucose + hexokinase (P/O ratio measured on this flask = 2·13); curve 3, 10^{-4} M DNP; curve 4, 0·0033 M ADP. (From Holton *et al.*, 1957. Reproduced by permission of the Editors, *Biochem. J.*)

occur before the introduction of phosphate into the reaction scheme, i.e. it must involve A ~ I, or X ~ I, rather than X ~ P. Since A ~ I is excluded by the considerations just mentioned, it is concluded that DNP promotes the hydrolysis of X ~ I.

The DNP-stimulated ATPase reaction is explained by the reaction sequence:

$$\text{ATP} + \text{X} \rightleftharpoons \text{X} \sim \text{P} + \text{ADP} \qquad (4)$$

$$\text{X} \sim \text{P} + \text{I} \rightleftharpoons \text{X} \sim \text{I} + \text{P}_\text{i} \qquad (5)$$

$$\text{X} \sim \text{I} + \text{H}_2\text{O} \xrightarrow{\text{(DNP)}} \text{X} + \text{I} \qquad (6)$$

$$\textit{Sum reaction}\ \text{ATP} + \text{H}_2\text{O} \xrightarrow{\text{(DNP)}} \text{ADP} + \text{P}_\text{i}$$

A comparison of the stimulation of the ATPase by DNP (pK = 4·1) and *p*-nitrophenol (PNP; pK = 7·2) at different pH's suggests that the phenolate ion is active rather than the undissociated phenol (de Ronden, 1958, unpublished).

All three phosphorylation steps of the respiratory chain are uncoupled by DNP. A study of the pH-activity curves of the ATPase of liver and heart mitochondria under various conditions led to the conclusion that there are three different DNP-stimulated ATPases, characterized by pH optima at 6·3, 7·4 and 8·5 (Myers and Slater, 1957*a*). These different enzymes have different sensitivities to DNP, the 6·3 enzyme being the most sensitive, and the 8·5 enzyme the least. A study of the pH-activity curves of oxidative phosphorylation (Hülsmann and Slater, 1957) led to the conclusion that the 8·5 enzyme was associated with $X \sim I_1$ (the intermediate in the phosphorylation between DPNH and fp), the 7·4 enzyme was associated with $X \sim I_2$ (the intermediate in the phosphorylation between cytochromes *b* and *c*), and the 6·3 enzyme was associated with $X \sim I_3$ (the intermediate in the phosphorylation at the oxygen end of the respiratory chain). These results are summarized in Table I.

Table I

Properties of I's

I	*Phosphorylative step of respiratory chain*	*pH optimum of reaction* $X \sim I + ADP + P_i \rightleftharpoons X + I + ATP$	[*DNP*] *required for 50 per cent maximum stimulation of ATPase* M
I_1	$DPNH + H^+ + fp \rightarrow DPN^+ + fpH_2$	8·5	6×10^{-5}
I_2	$2\,b^{\cdot\cdot} + 2\,c^{\cdot\cdot\cdot} \rightarrow 2\,b^{\cdot\cdot\cdot} + 2\,c^{\cdot\cdot}$	7·4	$1{\cdot}8 \times 10^{-5}$
I_3	$2\,a^{\cdot\cdot} + \frac{1}{2}\,O_2 + 2\,H^+ \rightarrow 2\,a^{\cdot\cdot\cdot} + H_2O$	6·3	8×10^{-6}

The conclusion, drawn from the pH-activity curves of the ATPase reaction (Myers and Slater, 1957*a*) and of oxidative phosphorylation (Hülsmann and Slater, 1957), that the DNP-stimulated ATPase with pH optimum at 6·3 is associated

with the phosphorylation coupled with the cytochrome oxidase reaction has been supported by experiments which have shown that this phosphorylative step is more sensitive to low concentrations of PNP (Table II).

Table II

Uncoupling of oxidative phosphorylation by *p*-nitrophenol

Rat heart sarcosomes; pH 7. It should be noted that the sarcosomes were not given a preliminary hypotonic pretreatment in the experiments with ascorbate and cytochrome *c* (see Slater, 1955).

Expt.	*Substrate*	*PNP* (M)	*P/O*	*Uncoupling (per cent)*
1	Glutamate	—	2·36	
	Glutamate	5×10^{-5}	1·71	28
	Succinate	—	1·35	
	Succinate	5×10^{-5}	0·85	37
	Ascorbate + cytochrome *c*	—	0·28	
	Ascorbate + cytochrome *c*	5×10^{-5}	0·15	47
2	Glutamate	—	1·78	
	Glutamate	10^{-4}	0·90	49
	Succinate	—	0·99	
	Succinate	10^{-4}	0·28	72
	Ascorbate + cytochrome *c*	—	0·13	
	Ascorbate + cytochrome *c*	10^{-4}	0·01	92

Ageing of mitochondria

The oxidative phosphorylation system in mitochondria becomes "uncoupled" if the mitochondria are allowed to stand for a few hours at room temperature (Kielley and Kielley, 1951). The "uncoupling", as revealed by the ATPase activity, reaches a maximum if the aged mitochondrial suspension is then subjected to repeated freezing and thawing (Myers and Slater, 1957*a*). Fragmented mitochondrial preparations, such as the Keilin and Hartree heart-muscle preparation (Myers and Slater, 1957*a*) or the digitonin particle prepared from liver mitochondria by Cooper and Lehninger (1956), have similar properties (Purvis, 1959). Unlike intact mitochondrial preparations, these preparations require the addition of Mg^{2+} for optimal ATPase activity. DNP has no effect.

Since these preparations oxidize substrates in the absence

of inorganic phosphate, either $A \sim I$ or $X \sim I$ must be hydrolysed (unless the hydrogen transfer in reaction (1) can occur without the participation of I). The resemblances between the ATPase reaction in these preparations and the DNP-stimulated ATPase of liver mitochondria (Myers and Slater, 1957*a*) suggest that the same hydrolysis of $X \sim I$ occurs. However, there are sufficient differences to suggest that $X \sim P$ (or one of the $I \sim P$'s) may also be hydrolysed, by a Mg^{2+}-requiring system, so that, in addition to the ATPases described by reactions (4)–(6), one described by reactions (7)–(8) is possible (Myers and Slater, 1957*b*; Purvis and Slater, 1959):

$$\text{ATP} + \text{X} \rightleftharpoons \text{X} \sim \text{P} + \text{ADP} \qquad (7)$$

$$\text{X} \sim \text{P} + \text{H}_2\text{O} \rightarrow \text{X} + \text{P}_i \qquad (8)$$

$$\text{Sum reaction } \text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{P}_i$$

Indeed, this reaction sequence may constitute the DNP-insensitive ATPase which is found in freshly prepared liver mitochondria at high pH's (pH optimum, 9·4). It may also account for the high ATPase activity, measured in the presence of added Mg^{2+}, found with isolated sarcosomes. It remains an open question whether this activity in isolated sarcosomes is due to structural damage to the sarcosomes, during isolation, or is related to an energy-utilization reaction in the cell. It is possible that, *in vivo*, one or more of the energy-rich intermediates which are formed prior to ATP in reactions (1)–(3*b*) [i.e. $A \sim I$, $X \sim I$ or $X \sim P$ ($I \sim P$)] are utilized directly for some of the energy-requiring reactions in the cell (cf. Slater, 1953). In fact, it is possible that reaction (3*b*) is, *in vivo*, more important when it runs from right to left (thereby utilizing the ATP synthesized in glycolysis) than from left to right. In other words, the biochemist when measuring oxidative phosphorylation in isolated sarcosomes, might be measuring to some extent the reverse of an ATP-utilizing reaction, rather than an ATP-synthesizing reaction.

Some differences between the DNP-stimulated ATPase of

freshly prepared liver mitochondria and the ATPase of aged mitochondria, or mitochondrial fragments are:

(i) The Mg^{2+} requirement of aged mitochondria, which could be explained by loss of Mg^{2+} from the mitochondria during fragmentation.

(ii) Between pH's 6 and 8, fluoride inhibits the DNP-stimulated ATPase much more than the system in aged mitochondria (Myers and Slater, 1957*b*). This could be explained by assuming that the reactions (3*a*) and (5) involving $X \sim I_3$ and $X \sim I_2$ are sensitive to fluoride, but the hydrolysis of $X \sim P$ (or $I \sim P$) in aged mitochondria is insensitive. The sensitivity to fluoride of the $^{32}P_i$-ATP exchange reaction (Hülsmann, 1958, unpublished) is in agreement with this explanation.

(iii) $Na_2S_2O_4$ has no effect on the ATPase in freshly prepared mitochondria, but stimulates (with peak of activity at pH 7·4) in aged mitochondria. On the basis of the reaction schemes given above, it could be concluded that $Na_2S_2O_4$ stimulates the hydrolysis of $X \sim I_2$ (and perhaps also $X \sim I_3$) in aged mitochondria.

(iv) 10^{-3} M-DNP inhibits the 6·3 enzyme in fresh mitochondria, but has little effect on aged mitochondria. A possible explanation is that high concentrations of DNP inhibit the hydrolysis of $X \sim I_3$ (promoted by lower concentrations), but have no effect on the hydrolysis of $X \sim P$.

Polis and Shmukler (1957) and Pullman and Racker (1956) showed that it was possible to isolate from aged mitochondria a substance which uncoupled oxidative phosphorylation in fresh mitochondria. Polis and Shmukler (1957) isolated the naturally occurring uncoupling agent from liver mitochondria and purified it. They found that the purified preparation had the spectrum of a haem compound with only one band at 410 mμ when oxidized and 422 mμ when reduced. The reduced form combined with CO, with a slight shift to 418 mμ, and

with a considerable intensification of the band, while the oxidized form combined with cyanide, with a shift of the band from 410 mμ to 418 mμ. They gave the name "mitochrome" to this pigment.

The action of this mitochrome preparation has been studied by Hülsmann, Elliott and Rudney (1958) who found that the uncoupling activity could be separated from the haem compound by extraction with *iso*octane. The uncoupling activity was found quantitatively in the *iso*octane, while the spectrum of the haem compound (for which the name mitochrome is retained) was found unchanged in the water layer. The uncoupling activity of the unextracted mitochrome preparation was unaffected by the addition of reducing or oxidizing agents, or by the addition of cyanide or CO, which combined with mitochrome. Boiling the mitochrome preparation, or precipitation with trichloroacetic acid, did not destroy the uncoupling activity, which was found in the precipitate.

It is clear, then, that the uncoupling activity of Polis and Shmukler's mitochrome preparation is not due to the haem-protein mitochrome which it contains, but to a component, presumably lipid in nature, which is extractable by organic solvents from this preparation. However, it is possible that the lipid material is more than an accidental contaminant of mitochrome.

Hülsmann, Elliott and Rudney (1958) (see also Elliott, Hülsmann and Slater, 1959) found that the spectrum of partially purified preparations of cytochrome ($a + a_3$), containing cholate and perhaps hydrolytic enzymes, changed to that of mitochrome after standing at 4° for a few days. A preparation of cytochrome b (containing some cytochrome c_1) behaved in the same way. These preparations of cytochrome ($a + a_3$) and of cytochrome b which had turned into mitochrome yielded an *iso*octane extract with the same properties as the lipid extracted from Polis and Shmukler's mitochrome preparation. The original cytochrome preparations yielded much less of this uncoupling material (Table III).

Table III

EXTRACTION OF UNCOUPLING LIPID FROM FRESH AND AGED CYTOCHROME PREPARATIONS

Cytochrome preparations were extracted with *iso*octane, after storage for various times at 4°. After evaporation of the *iso*octane, the residue was dissolved in alcohol and a small sample of the alcoholic solution added to a reaction system measuring the ^{32}P-ATP exchange reaction in freshly prepared rat liver mitochondria.

Preparation extracted with isooctane	*Days at 0°*	*Exchange (μatoms P/mg. protein/hr.)*
None	—	6·1
Cytochrome ($a + a_3$)	0	4·7
	7	2·1
	10	1·5
Cytochrome *b*	0	3·0
	5	0·9

Thus, it appears that mitochrome is a haemprotein (or a mixture of haemproteins) derived from the cytochromes. There is a time correlation between the inactivation of the cytochrome *c* oxidase (in the case of the cytochrome ($a + a_3$) preparation), the disappearance of the cytochrome spectrum, the appearance of that of mitochrome and the extractability of the uncoupling substance by *iso*octane. This suggests the following transformation

$$\underset{\substack{|\\ \text{lipid}}}{\text{cytochrome}} \rightarrow \text{mitochrome} + \text{lipid}$$

The lipid is normally bound to cytochrome, and is liberated (and therefore becomes extractable by *iso*octane) during the transformation of cytochrome to mitochrome. It must be emphasized, however, that the possibility cannot be excluded that the appearance of extractable lipid and the transformation of cytochrome to mitochrome are two unrelated processes which take place during treatment of cytochrome preparations with hydrolytic enzymes.

Like that of the unextracted mitochrome preparation of Polis and Shmukler, the uncoupling activity of the lipid extracted by *iso*octane is counteracted by the addition of albumin. It appears that albumin firmly binds the substance.

This is interesting, because Polis and Shmukler (1957) found that mitochrome (the haemprotein) was bound so firmly to albumin that the two were not separated by electrophoresis. It seems likely that this binding occurred through the lipid uncoupling agent.

Pressman and Lardy (1952) isolated from microsomes a heat-stable, acetone-soluble fraction which stimulated the respiration of liver mitochondria in a medium deficient in phosphate acceptor, and stimulated the ATPase. The active substances were later identified as a mixture of long-chain fatty acids (Pressman and Lardy, 1955, 1956). Unsaturated fatty acids such as oleic acid were found to be especially active as uncoupling agents (Pressman and Lardy, 1956).

Chemical studies of the uncoupling lipid extractable by *iso*octane from mitochrome preparations have shown that it also is an unsaturated fatty acid.

It appears likely that the high ATPase and low phosphorylative activity of aged liver mitochondria are at least partly due to the liberation of the lipid uncoupling material. The pH-activity curve of the ATPase of liver mitochondria aged for 2 hours at 30° has two peaks, at 6·3 and 9 (Myers and Slater, 1957*a*). The same peaks of activity were obtained with liver mitochondria to which was added either oleic acid or an acid fraction isolated from the *iso*octane extract of the mitochrome preparation. Lower concentrations of the lipids stimulated only in the region of 6·3. The further activation of the ATPase of liver mitochondria, particularly in the 7·4 and 8·5 regions, which occurs when the suspension aged at 30° for 2 hours is subjected to freezing and thawing (Myers and Slater, 1957*a*), is probably not due to the liberation of the uncoupling lipid.

Possible control of metabolism by uncoupling agents

The isolation from mitochondria and from microsomes of substances which uncouple oxidative phosphorylation in freshly prepared liver mitochondria raises the question of the

possible function, *in vivo*, of such naturally occurring uncoupling agents. There are three possibilities which might be considered:

(1) The first possibility is applicable only to the uncoupling agent isolated from aged mitochondria. It is possible that when the lipid is bound to cytochrome as in the intact mitochondria, it is in some way involved in oxidative phosphorylation (possibly as I), but that when it is liberated from aged mitochondria and added back to freshly prepared mitochondria, it acts as an uncoupling agent.

(2) The second possibility, which is applicable to either uncoupling agent, is based on Lardy's (1952) suggestion that an uncoupling agent might offer a more sophisticated type of control mechanism than that provided by variation of the ADP concentration (cf. Lardy, 1955; Pressman and Lardy, 1956). Under certain circumstances the rate of respiration might be controlled by the rate at which one of the $X \sim I$'s reacts with P_i and ADP [reactions (3*a*) and (3*b*)]. If an uncoupling agent specific for this $X \sim I$ were present, this rate-limiting step would be accelerated. This would mean that the rate of hydrogen transfer, and therefore the rate of synthesis of ATP would be increased in the other still coupled steps of the respiratory chain. This increased rate of synthesis of ATP in these two steps might more than compensate for the loss of ATP in the uncoupled step. The feasibility of this sort of control mechanism was demonstrated by Slater and Lewis (1954) under rather special (and probably freak) circumstances, using sarcosomes isolated from the thoracic muscle of the blowfly.

In the experiment illustrated in Fig. 4, the ATP synthesized was immediately utilized for the synthesis of hexose monophosphate (HMP) from glucose. Respiration was not, therefore, limited by the supply of ADP. It may be seen that increasing concentrations of DNP caused an increase in the rate of oxygen uptake, and particularly of the α-ketoglutarate utilized. With low concentrations of DNP, the amount of HMP formed was decreased, because DNP inhibited the

synthesis of ATP in the respiratory chain. The residual synthesis of HMP is due to the fact that the first step of the α-ketoglutaric oxidase system is coupled with phosphorylation in a reaction which is not sensitive to DNP. With increasing concentrations of DNP, the rate of hydrogen transfer increases

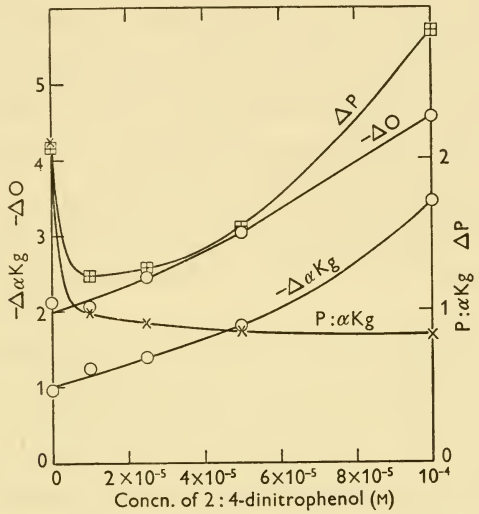

Fig. 4. Effect of increasing concentrations of DNP on the rate of respiration of blowfly thoracic-muscle sarcosomes, and on the accompanying esterification of inorganic phosphate. ΔP = esterified phosphate (μmoles), $-\Delta O = O_2$ uptake (μatoms), $-\Delta\alpha Kg$ = αketoglutarate oxidized (μmoles). (From Slater and Lewis, 1954. Reproduced by permission of the Editors, *Biochem. J.*)

and consequently the amount of phosphorylation associated with the first step also increases, until eventually the increased phosphorylation in this step more than compensates for the complete inhibition of phosphorylation in the rest of the chain. The net result is that DNP increases the rate of phosphorylation, although the efficiency of the conversion of oxidation energy to the synthesis of ATP is of course decreased.

This experiment illustrates the feasibility of Lardy's

theory, but it remains to be determined whether either of the naturally occurring uncoupling agents works so selectively as to cause an increase in the rate of synthesis of ATP, in the presence of excess ADP. Incidentally, the sarcosomes used in the experiment described in Fig. 4 probably contained the uncoupling agent which is isolated from mitochrome preparations. This is indicated by the marked increase in the P : O ratio brought about by the addition of serum albumin (Lewis and Slater, 1954), first found by Sacktor (1954) to be useful in the isolation of insect sarcosomes. Albumin has no appreciable effect on oxidative phosphorylation or on the ^{32}P-ATP exchange reaction in rat heart sarcosomes.

(3) The third possibility is that an uncoupling agent might be concerned in the utilization by the cell of the energy of one of the intermediates of oxidative phosphorylation (see p. 67).

Possible nature of the I compounds

The exact location of the phosphorylation steps in the respiratory chain is not yet established. However, it seems very likely on thermodynamic grounds (Slater, 1959) that the reaction between DPNH and fp is coupled with the synthesis of ATP.

According to equation (1), the first energy-rich intermediate formed in the reaction between DPNH and fp would be DPN $\sim$ I_1. However, all the experiments described to date could equally well be explained by the supposition that I combines with the reduced product (BH_2) of reaction (1), as preferred by Chance and Williams (1956). If this were the case, the product of reaction between DPNH and fp would be $fpH_2 \sim I_1$ rather than DPN $\sim$ I_1. Neither equation (1) as written nor this modification could account for the formation by this reaction of DPNH $\sim$ I, which has been postulated by Chance and Williams (1956).

Purvis (1958) has recently obtained evidence for the presence in liver mitochondria of a form of DPN which has the properties predicted for DPN $\sim$ I_1. He measured the amounts

of DPN and DPNH by specific enzymic fluorometric methods applied to acid and alkaline extracts of liver mitochondria after different times of incubation at 30°, in the presence of air, inorganic phosphate or ADP. After about 5 minutes' incubation, all the DPNH initially present had been oxidized, but the amount of DPN appearing greatly exceeded the amount of DPNH oxidized (i.e. after 5 minutes, the DPN content exceeded the amount of DPN + DPNH initially present). The extra DPN must have been derived from some precursor. DNP was found effective in place of ADP or inorganic phosphate, but ATP was ineffective. This strongly suggests that the precursor is connected with oxidative phosphorylation and, in fact, it has the properties expected of DPN $\sim I_1$. Mean values (μmoles/g. protein) for the concentrations of the various forms in rat liver mitochondria were: DPN, 1·05; DPNH, 1·60; DPN $\sim$ I, 1·67. The value for DPN $\sim$ I agrees closely with the estimate by Eisenhardt and Schrachinger (1958) of the amount of high-energy compounds in liver mitochondria (1·5 μmoles/g. protein) obtained by determining the rapid increase in ATP which occurs immediately after the addition of ADP to liver mitochondria.

It should be emphasized that the proposed DPN $\sim$ I has not yet been directly identified in either the acid or alkaline extracts of mitochondria*. Its existence is inferred from the increased (DPN + DPNH) which is found after incubation with ADP, inorganic phosphate, or DNP.

The concentration of the supposed DPN $\sim$ I in liver mitochondria after various treatments was measured by determining the difference between the total diphosphopyridine nucleotide content (measured by incubating untreated mitochondria at 30° with ADP or DNP, followed by determination of the DPN content) and the sum of the DPN and DPNH contents measured on acid and alkaline extracts, respectively, of the treated mitochondria. The concentration

* *Note added in proof.* Dr. Purvis has now obtained evidence of a substance in alkaline extracts of fresh mitochondria which yields DPN on the addition of aged mitochondria to this neutralized extract.

of DPN ~ I determined in this way increased by incubation with ADP, nicotinamide and amytal (0·8 mM). Subsequent dilution and addition of DNP caused the formation of more DPN.

These interconversions of the various forms of DPN can be explained on the basis of reactions (1)–(3*b*), and (6), in which AH_2 is DPNH and B is fp. Addition of ADP or DNP liberates I_1 from its combination with X and promotes the oxidation of DPNH by equation (1). The simultaneous liberation of X promotes the formation of DPN from DPN ~ I_1. The accumulation of DPN ~ I_1 in the presence of ADP and low concentrations of amytal suggests that amytal acts on reaction (2). It might be added that these explanations require that the intermediate is DPN ~ I, not DPNH ~ I.

The effects of addition of substrate (in the presence of oxygen, but absence of phosphate acceptor) on the concentrations of the three forms of diphosphopyridine nucleotide are particularly interesting. The addition of α-ketoglutarate, glutamate or succinate caused a decrease of DPN, while the DPNH either decreased or did not change (see Table IV). In other words, DPN ~ I increased. The effects of α-ketoglutarate and glutamate are in agreement with expectations according to the following sequence (where SH_2 = substrate; S = product):

$$SH_2 + DPN \rightleftharpoons S + DPNH \qquad (9)$$

$$DPNH + fp + I_1 \rightleftharpoons DPN \sim I_1 + fpH_2 \qquad (10)$$

$$fpH_2 + \tfrac{1}{2}\,O_2 \rightarrow fp + H_2O \qquad (11)$$

$$DPN \sim I_1 + X \rightleftharpoons DPN + X \sim I_1 \qquad (12)$$

Table IV shows that β-hydroxybutyrate caused an increase of both DPN and DPNH, with consequent decrease of DPN ~ I. This is unexpected, but could be explained if this substrate has a slight uncoupling activity.

The decrease of DPNH found after the addition of succinate is in contrast to the conclusion drawn by Chance (1956), on the basis of direct spectrophotometric observations of liver

mitochondria. Chance found that the addition of succinate to aerobic mitochondria caused an increase of the extinction at 340 mμ, measured with respect to the extinction at 374 mμ, and concluded that the spectral change corresponded to the reduction of DPN. If, however, DPN ~ I has the same

Table IV

Effect of addition of substrates to liver mitochondria on level of forms of diphosphopyridine nucleotide

Substrate was allowed to react with liver mitochondria suspended in 0·18 M sucrose for 90 seconds at 0°. Final volume, 0·5 ml. No change in DPN or DPNH contents occurred when suspension was incubated in absence of substrate.

		Diphosphopyridine nucleotide (μmoles/g. protein)		
Expt.	*Substrate*	*DPN*	*DPNH*	*DPN ~ I**
1	None	1·78	1·56	1·10
	α-Ketoglutarate (10 μmoles)	0·70	1·40	2·34
	Glutamate (20 μmoles)	1·28	1·56	1·60
2	None	1·60	1·94	1·18
	Succinate (20 μmoles)	0·82	1·77	2·13
3	None	1·73	2·51	0·70
	Succinate (20 μmoles)	0·74	2·16	2·00
4	None	1·47	1·28	1·44
	†DL-β-hydroxybutyrate (15 μmoles)	1·86	2·00	0·33

* DPN ~ I = (Total DPN obtained after incubation at 30° with DNP) *minus* (DPN + DPNH).
† 0·005 M-$MgCl_2$ also added.

spectral properties as other addition compounds of DPN, it would also be expected to absorb in the same region as DPNH. Chance's spectrophotometric method might not, therefore, distinguish between DPNH and DPN ~ I.

Chance's (1956) report that succinate was able to reduce DPN was rather unexpected, because DPN is not a member of the succinate chain. Chance (1958) suggested that "this reduction was brought about by a reversal of electron transport through cytochrome *b* and flavoprotein that leads to a reduction of pyridine nucleotide. This reaction is mediated by endogenous high-energy intermediates present in the

phosphorylating mitochondria and upon which the reaction depends to overcome the thermodynamic difficulties".

The finding (Purvis, 1958) that succinate does not reduce DPN, but instead causes the accumulation of DPN $\sim$ I can be explained, without suggesting a reversal of the respiratory chain, by assuming that either $X \sim I_2$ or $X \sim I_3$ formed in reactions (1) and (2), with succinate as substrate, can react with I_1 [possibly via $X \sim P$ (see Purvis, 1958)], e.g.

$$X + I_2 \xrightarrow{\text{[reactions (1) and (2)]}} X \sim I_2 \qquad (13)$$

$$X \sim I_2 + I_1 \rightleftharpoons X \sim I_1 + I_2 \qquad (14)$$

$$X \sim I_1 + DPN \rightleftharpoons DPN \sim I_1 + X \qquad (15)$$

Sum reaction $DPN + I_1 \xrightarrow{\text{[reactions (1) and (2)]}} DPN \sim I_1$

In other words, the energy-conservation reactions linked with the oxidation of succinate are used to synthesize the energy-rich compound $DPN \sim I_1$ from its constituents.

This explanation also takes account of the inhibition by antimycin of the reduction of DPN by succinate (Chance and Hollunger, 1957), since antimycin inhibits reaction (13), and the inhibition by amytal, if the latter inhibits reaction (15). The inhibition by DNP (Chance and Hollunger, 1957) is also easily explained, since it promotes the hydrolysis of $X \sim I_2$ and $X \sim I_1$.

Importance, *in vivo*, of the control of respiratory rate by processes related to oxidative phosphorylation

Equations (1)–(3*b*) predict that, in the absence of reactions liberating I from one of its bound forms, reaction between AH_2 and B is inhibited, and that this inhibition would be relieved by the addition of ADP and inorganic phosphate. Chance and Williams (1956) showed that addition of ADP to liver mitochondria relieved the inhibition of respiration and caused the oxidation of all components of the respiratory

chain below cytochrome *a*, and the reduction of cytochrome *a*. Chance and Baltscheffsky (1958) have found that rat heart sarcosomes behave similarly. It is, therefore, of very considerable interest that Chance and Connelly (1957) have found that stimulation of frog sartorius muscle causes the oxidation of the DPNH and that Weber (1957) has found that cytochrome *b* was oxidized and cytochrome a_3 reduced. This is very suggestive that the increased respiration brought about by muscular stimulation proceeds through the same type of mechanism as formulated in equations (1)–(3*b*). In fact, Chance and Connelly calculated the amount of ADP reaching the sarcosome, on the assumption that the two processes were completely analogous.

However, it must be emphasized that the fact that stimulation of the muscle has the same type of effect as addition of ADP to liver mitochondria is no proof that ADP is indeed formed by a muscular contraction. The same result would be obtained if muscular contraction were associated with a hydrolysis of $A \sim I$, $X \sim I$ or $X \sim P$, instead of the hydrolysis of ATP to ADP. Indeed, Chance and Williams (1956) have demonstrated that the uncoupling agent, dicoumarol, which is believed to promote the hydrolysis of $X \sim I$, has the same sort of effect as the addition of ADP. This is an important point, because the chemical studies of Fleckenstein and co-workers (1954) and Mommaerts (1954) have not demonstrated the breakdown of ATP during muscular contraction. The very close topographical arrangement between the sarcosome and the myofibril (Cleland and Slater, 1953) suggests the possibility that $A \sim I$, $X \sim I$ or $X \sim P$ might be directly utilized for the energy of muscular contraction, without the intermediate formation of ATP (see above). This question will no doubt be settled by direct chemical analysis of the changes in muscle which occur on muscular contraction.

In any case, there is no essential disagreement between the results of Fleckenstein and co-workers (1954) and Mommaerts (1954), on the one hand, and Chance and Connelly (1957) on the other, since the amount of ADP calculated by

the latter (and this amount might be overestimated, but not underestimated) was less than the sensitivity of the methods used by Fleckenstein and co-workers (1954) or Mommaerts (1954). The three groups agree that the amount of ADP formed was only a very small fraction of the value expected on the assumption that the hydrolysis of ATP provides the energy for the contraction of the muscle.

Although Chance and Connelly's (1957) results cannot yet be taken to establish that the respiration of muscle is controlled by the concentration of ADP, they do provide good support for the view that either the breakdown of ATP, or of intermediates in the oxidative phosphorylation reaction, are an important mechanism for the control of respiration, *in vivo*.

Other evidence pointing in the same direction is: (1) The increased metabolism of animals after administration of DNP or related compounds (Cazeneuve and Lepine, 1885; Mathews and Longfellow, 1910; Heymans and Bouchaert, 1928). Ehrenfest and Ronzoni (1933) found that DNP stimulated the respiration of isolated frog muscle sevenfold. According to the formulation of equations (1)-(3*b*), DNP might not be expected to have very much effect on the rate of respiration, if it is added to a system already supplied with ADP as in actively metabolizing muscle, even though it will stop the synthesis of ATP. Under these circumstances, the rate of respiration is governed by the activity of the enzymic system bringing about reaction (1). If, however, DNP is added to a system corresponding to a resting muscle, in which respiration is largely blocked by the fact that $X \sim I$ is not broken down, it will cause the same stimulus of respiration as is brought about by muscular activity. Since, however, the energy made available by respiration is not utilized under these conditions, it will appear as heat. In fact, it was known to pharmacologists, long before the biochemical site of action of DNP was known, that animals receiving a toxic dose of dinitrophenols developed a very high temperature (Cazeneuve and Lepine, 1885).

Vitamin E-deficient animals also show an increased rate of respiration and heat production. This can also be demonstrated in the dystrophic muscles, isolated from the deficient animals (Victor, 1934). It appears that the dystrophic muscle has lost its respiratory-control mechanism. This may possibly reflect structural damage to the mitochondria. However, the fact that the increase of respiration occurs well in advance of the morphological degeneration during the course of avitaminosis E, and the rapidity with which the heightened respiration of the isolated muscle is suppressed soon after the administration of vitamin E suggest the possibility that the vitamin might be involved more specifically. This is currently under investigation.

(2) The studies of McIlwain and his co-workers (see McIlwain, 1955) have shown that the increase of the rate of respiration and glycolysis which occurs when brain slices are electrically stimulated is accompanied by the hydrolysis of creatine phosphate. Stimulation caused no increase in the rate of respiration in the presence of DNP (McIlwain and Gore, 1951). Kratzing (1952) obtained similar results with rat diaphragm.

Conclusion

There is now good reason to conclude that respiration *in vivo* is to a large extent controlled by the concentration of energy-rich intermediates of oxidative phosphorylation, named here $A \sim I$, $X \sim I$ and $X \sim P$ (or $I \sim P$). Respiration cannot proceed unless A, the respiratory-chain component, is liberated from its inhibited form, $A \sim I$. This can be brought about by reaction of $X \sim I$ with ADP, or by hydrolysis of any of the intermediates. The symbol I was introduced by Chance and Williams (1956), replacing C in the earlier formulation (Slater, 1953), in order to emphasize this inhibition. This was a useful change of nomenclature, since it directed attention to the analogies between inhibition of respiration associated with phosphorylation and the more commonly studied respiratory inhibitors, such as antimycin.

However, one has to be careful in extending the use of the word inhibitor too far, because I in equation (1) is necessary for respiration. In fact, removal of I would lead to inhibition of respiration. It is possible that inhibition of respiration by antimycin is due to combination with I_2 (Hülsmann, 1958).

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DISCUSSION

Krebs: I noticed that you had very large quantities of DPN∼I. What you determined was intramitochondrial DPN?

Slater: We determined what is found in an acid extract in order to determine the DPN, and what is found in an alkaline extract in order to determine the DPNH.

Krebs: That implies that this derivative is rather stable in acid and in alkali.

Slater: It does not liberate DPN or DPN∼I in acid or alkali. One would expect it to be stable in alkaline extract if it were something like a DPNH.

Lynen: It could be decomposed during the alkaline and acid extraction. You pointed out that the sum of DPN + DPNH is lower at the beginning than after incubation.

Slater: Yes, it could be decomposed during the acid or alkaline extraction.

Krebs: But it becomes stable when it is attached to the ~.

Slater: One determines DPN and DPNH, but more total DPN is found after incubating mitochondria.

Krebs: That may mean either that more is liberated or more is available, or that some of it has been destroyed in the initial material. In that case it would mean that it would stabilize the initial material. If in your ordinary determination you had some losses, you would get a certain lower value than you would get if you did not have these losses and, therefore, the bound DPN would prevent losses, if it were more stable.

Slater: These procedures can be controlled and we do not get losses with DPN or DPNH. The recoveries of DPN and DPNH are quantitative.

Potter: Prof. Slater, when you carry out the incubation of mitochondria, do you then centrifuge them down and treat the pellet with acid or alkali, as the case may be, or do you work on the whole incubation mixture?

Slater: We work on the whole incubation mixture.

Potter: So it is the sum of what is extractable from the mitochondria plus what may have leaked out into the medium.

Slater: Yes, it is the total. The rather thick mitochondrial suspension in sucrose is deproteinized at various times.

Racker: How do you explain that TPN~I goes up? Do you visualize this as a transfer reaction from DPN~I to TPN~I?

Slater: Yes, that would be one possibility. However, Dr. Purvis has not yet studied the effects of amytal or of substrate on TPN~I. These are the substances which cause DPN~I to go up. Therefore, the formation of extra TPN~I has not yet been demonstrated in the same way as the formation of extra DPN~I. What has been established is that incubation of mitochondria with ADP, phosphate or DNP, causes the appearance of extra TPN as well as of extra DPN. We interpret this in terms of a breakdown of TPN~I as well as of DPN~I. TPN~I could be formed by reaction of DPN~I with TPN, a sort of "pyridine nucleotide trans-I-ase" reaction, as you suggest.

Hess: What is the stoichiometrical relationship between the ADP that you added and the amount of DPN~I which appeared? Also, I would like to know whether it is excluded that there is a *de novo* synthesis of DPN.

Slater: The extra DPN appears very quickly—within a few minutes. This occurs in the presence of ADP, inorganic phosphate or DNP, but not ATP. If something were being synthesized, the opposite result might be expected; one would expect to get it synthesized in presence of ATP, but certainly not in presence of DNP. Although DPN is not supposed to be synthesized in mitochondria, but in the nuclei, this would not by itself be conclusive since there might be another unsuspected system for the synthesis of DPN in the mitochondria. Our reason for thinking that this is not the case is that the formation of DPN does not take place with ATP whereas it does with DNP. The process has all the characteristics of a breakdown of a compound con-

taining DPN, rather than the synthesis of DPN from substances of smaller molecular weight.

Hess: How much ADP do you need?

Slater: If ADP alone is added to mitochondria, there is half maximal appearance of extra DPN (a sort of K_m) at 200 μM (2×10^{-4} M), whereas it occurs at 70 μM if ADP is added in presence of 2 mM inorganic phosphate.

Lynen: Prof. Slater, you talk about concentrations of ADP and phosphate added, but what Dr. Hess wants to know is whether you can titrate the DPN∼I compound. If your equations are correct, then you would have to add to 1 μmole of DPN∼I, 1 μmole of ADP and inorganic phosphate.

Slater: We have not yet tried to titrate DPN∼I with ADP. Its average concentration, determined from the amount of extra DPN formed on the addition of excess ADP, is 1·67 μmoles/g. of protein. That comes out within 10 per cent or so of the value reported from Prof. Chance's laboratory (Eisenhardt and Schrachinger, 1958, *loc. cit.*), which was determined from the amount of ATP which is rapidly synthesized on the addition of ADP, which is really a titration of mitochondria for its ∼ content.

Chance: Prof. Slater has interpreted his experimental results on the assumption that a compound of oxidized DPN is converted, in the presence of ADP and inorganic phosphate, into free DPN which can then be assayed by the alcohol dehydrogenase.

$$\text{(1)} \qquad \text{DPN}\sim\text{I} \xrightarrow[\text{X}]{\text{ADP, P}_i} \text{DPN} + \text{ATP} + \text{X} + \text{I}$$

Our interpretation of spectroscopic studies of intramitochondrial pyridine nucleotide suggests that a DPNH compound (DPNH∼I) is converted to DPN according to the following equations, which express what we term the state 4-to-3 transition or the quiescent-active state transition:

$$\text{(2)} \qquad \text{DPNH}\sim\text{I} \xrightarrow[\text{X}]{\text{ADP, P}_i} \text{DPNH} + \text{ATP} + \text{X} + \text{I}$$

$$\text{(3)} \qquad \text{DPNH} + \text{rfp} + \text{H}^+ \longrightarrow \text{fp} + \text{DPN}^+$$

$$\text{(4)} \qquad \text{DPNH}\sim\text{I} + \text{rfp} + \text{H}^+ \xrightarrow[\text{X}]{\text{ADP, P}_i} \text{DPN}^+ + \text{fp} + \text{ATP} + \text{X} + \text{I}$$

The experimental data presented by Prof. Slater are consistent with equations 2, 3 and 4: addition of ADP produces DPN^+; addition of dinitrophenol produces DPN; and addition of amytal diminishes DPN^+ by blocking equation 3. The spectrophotometric traces indicate that the "new form" of pyridine nucleotide is reduced, not oxidized. However, Prof. Slater's suggestion that the opposite conclusion is true raises

this question: is there, in the mitochondria, a form of DPN which we have mistaken for DPNH?

First, an absorption peak at 340 mμ in the mitochondrial state (state 4) which contains a maximal concentration of Slater and Purvis' DPN~I compound is clearly shown by the spectroscopic data of Fig. 1A. ADP addition diminishes absorption at 340 mμ. Similar results have been obtained for heart muscle mitochondria. It is a justifiable conclusion that the form of pyridine nucleotide affected by ADP absorbs at 340 mμ to within an accuracy of 1–2 mμ. Also, the shape of the band is very similar to that recorded for a solution of DPNH.

Two compounds of oxidized DPN have absorption bands in this region. The more relevant for our consideration is that of DPN with

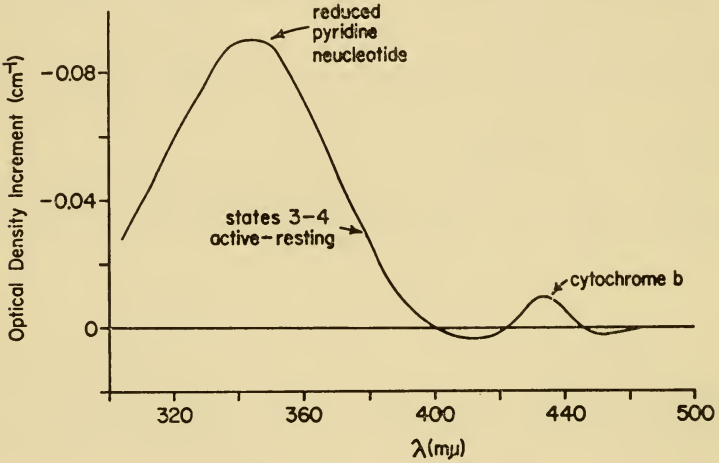

FIG. 1A (Chance). (Expt. no. 661b)

glyceraldehyde-3-phosphate dehydrogenase, which has an absorption maximum at 365 mμ; this is displaced 25 mμ from the absorption maximum of reduced DPN and is a much broader band. The second is the compound of oxidized DPN and cyanide, which has an absorption maximum at 325 mμ. High concentrations and pH values are necessary for its formation, and it is therefore doubtful that it could be formed under physiological conditions. I do not know of a DPN compound that absorbs at 340 mμ.

Racker: Is it not correct that the dihydroxyacetone compound with DPN absorbs at 340 mμ?

Chance: Yes, this was actually mistaken for the reduced form.

[*Note added in proof.* The dihydroxyacetone compound is actually a reduced form since it is at the oxidation level of reduced DPN (Burton, R. M., San Pietro, A., and Kaplan, N. O. (1957). *Arch. Biochem.*, 70, 89).]

Another criterion for the oxidation state of pyridine nucleotide in mitochondria which we have recently applied in some detail (Chance, B., and Baltscheffsky, H. (1958). *J. biol. Chem.*, **733**, 736) is that the oxidized form of DPN has negligible fluorescence and the reduced form has intense fluorescence, with an emission maximum at 470 mμ for the free form and at 443 mμ for the bound form (Boyer, P. D., and Theorell, H. (1956). *Acta chem. scand.*, **10**, 447; Duysens, L. N. M., and Amesz, J. (1957). *Biochim. biophys. Acta*, **24**, 19). We have recently investigated the fluorescence of mitochondria in the absence of ADP (state 4), and find a maximum at 443 mμ which diminishes upon addition of ADP (Fig. 1B).

In summary, spectrophotometric and fluorometric data indicate the

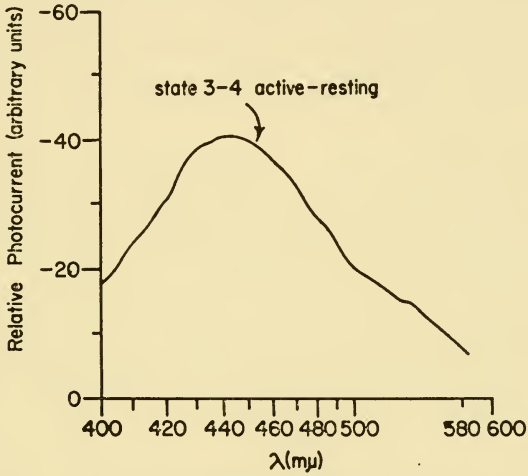

FIG. 1B (Chance). (Expt. no. 777)

presence of a form of reduced pyridine nucleotide in the mitochondria, the concentration of which is diminished by ADP and increased by amytal. This form, therefore, appears to be identical with the compound which Prof. Slater is attempting to assay enzymically. Unless he can present physical data proving that he is studying a form of oxidized DPN which has the fluorescence and absorption properties of reduced DPN, it seems appropriate that he consider the possibility that he is studying the DPNH~I compound rather than the DPN~I compound as an explanation for his experiment.

[*Note added in proof.* For some time there has been evidence that the DPNH~I compound of mitochondria may not be as active towards certain dehydrogenases as is free DPNH: "Upon addition of acetoacetate to the mitochondria in the resting state, 4, the extent of oxidation of DPNH is very small compared with that which is obtained upon addition of acetoacetate to state 3." (Chance, B., and Williams, G. R. (1956). *Advanc. Enzymol.*, **17**, 107).]

Slater: At the present stage of our work, we should not like to make a final decision as to whether our compound is a DPN∼I or DPNH∼I compound. There are many observations which we and others have made which are easier to explain on the basis of a DPN∼I compound than of a DPNH∼I compound. But I should not say that they were conclusive. At the moment we should say that we have evidence for an intermediate precursor of DPN or DPNH. I would not like to go so far as to say that this is certainly a DPN and not a DPNH compound. We hope to settle the question by isolating the compound.

As Dr. Racker has pointed out, it is certainly possible to have a DPN∼I compound which absorbs maximally at 340 mμ.

Siekevitz: Some evidence has been published that the initial phosphorylation takes place between DPNH and flavoprotein by means of a direct phosphorylation of the flavoprotein, and that a loose complex might be formed between DPNH and flavoprotein if inorganic phosphate is around (Löw, H., Siekevitz, P., Ernster, L., and Lindberg, O. (1958). *Biochim. biophys. Acta*, **29**, 392). I wonder if your inhibited compound might either be a DPNH phosphate—a cyclic phosphate—or a DPNH flavoprotein.

Slater: Dr. Ernster's theory, as I understand it, is that a reduced flavoprotein phosphate is the intermediate; and that is rather different from what we are suggesting here. He suggests a reaction of DPNH plus the flavoprotein plus inorganic phosphate which gives reduced flavoprotein phosphate plus oxidized DPN; so he has no intermediate of the DPN, which is what we are studying.

Siekevitz: He also thinks that initially there is a charged ion complex formation between DPNH and flavoprotein.

Slater: On the evidence of these experiments alone, anything like that is possible. We do not think it is so, on the basis of other experiments which lead us to believe that phosphate comes into the series of reactions rather later.

Lehninger: In the course of our own work on the mechanism—as I will explain later—we have also been searching for "high-energy" derivatives of DPN. One point which came to our attention very forcibly is that not all the DPN molecules in the mitochondrion may be of the same chemical species (Devlin, T. M. (1959). *J. biol. Chem.*, in press; Gamble, J. L., Jr., and Lehninger, A. L. (1956). *J. biol. Chem.* **223**, 921). Prof. Chance has also presented evidence that there is some functional compartmentation. Our evidence derives from studying the digitonin fragments of the mitochondrial membrane. There, the bound DPN seem to have quite different characteristics from the total DPN of intact mitochondria. This brings up the suggestion that possibly only a few DPN molecules out of the large number in mitochondria may be directly connected with each respiratory chain; the others might feed into it via a DPNH—DPN transhydrogenase. The bound DPN of the submitochondrial fragments is not discharged by phosphate.

Prof. Slater, have you followed the extramitochondrial DPN during some of your experiments? Hunter found discharge of DPN from mitochondria with phosphate, and recently he reported that he can rebind

this DPN again (Hunter, F. E., Jr., Shutz, B., Malison, R., and Atchison, A. (1958). *Fed. Proc.*, **17**, 247). Do you think there is any association between this discharge phenomenon and the conversion of DPN∼I to DPN?

Slater: I am not familiar with this work of Hunter which you mention. We have not determined whether some of the DPN or DPNH has gone outside the mitochondria or whether it all remains inside. We shall certainly test this.

Racker: Prof. Lehninger, do these different DPN's react differently with various dehydrogenases?

Lehninger: There certainly is a great difference in reactivity toward β-hydroxybutyric acid and malic dehydrogenases. In the digitonin particles there is less than one mole of bound DPN per mole of cytochrome *a* and this one is apparently concerned in phosphorylation. The intact mitochondria have something like 40 moles of DPN. It is possible therefore that you cannot see that one DPN molecule very well in a pool of 40 when you are studying intact mitochondria spectrophotometrically; the "pools" of DPN may have different functions.

Slater: In our experiment, what we obtain is the appearance of extra DPN, more than we have at zero time. We get this with ADP, inorganic phosphate or DNP, but not with ATP. That is what links it with oxidative phosphorylation. We were not looking for a DPN intermediate of oxidative phosphorylation. It was found accidentally while Dr. Purvis was trying to deplete mitochondria of DPN for his work on pyridine nucleotide transhydrogenase.

Holton: In reference to one of your mechanistic slides* with DPNH, the total mechanism is very complex. Does your evidence for DPN∼I mean that one of these intermediate steps can be struck out from your mechanism? For instance, the action of DNP could well be in breaking this DPN∼I_1 down. You have introduced another energy-rich inhibited compound. Is there a possibility of simplification by cutting out one step?

Slater: The reason for introducing this extra step was that the ATPase reaction, which involves this X∼I, was not affected by the state of oxidation and reduction of the respiratory chain. We had to take components of the respiratory chain out of the ATPase reaction, and our reasons for doing that still remain. Prof. Chance introduced the X for a kinetic reason (Chance, B., and Williams, G. R. (1956). *Advanc. Enzymol.*, **17**, 65). Certainly, we would still retain the necessity for the X.

Holton: We have recently come upon some independent evidence that the compound whose breakdown is catalysed by DNP is formed by a sequence of at least two reactions. Dr. Beechey, in our laboratory, has been studying heart sarcosomes possessing respiratory control (Beechey, R. B. (1959). *Biochim. biophys. Acta*, in press) using the polarometric method of Chance and Williams (1955, *Nature* (*Lond.*), **175**, 1120). He has found that their respiration attains approximately the same rate with ADP as with optimal concentrations of DNP or dicoumarol; this suggests that the same reaction is limiting the rate with the

* [Not submitted for publication.—EDS.]

two reagents. By contrast, when magnesium is added the respiration becomes considerably faster than is found with ADP or DNP, and respiratory control is entirely removed. Loss of respiratory control indicates breakdown of an intermediate in the energy-transfer chain, but the fact that a higher respiration rate is observed suggests that the intermediate concerned cannot be identical with the one whose breakdown is catalysed by DNP, and that it is formed by a reaction which comes earlier in the energy-transfer chain. Alternative explanations of the magnesium effect involve its action at more than one point in the reaction sequence and for that reason seem to me to be less likely. It may be noted that this evidence is distinct from the kinetic evidence adduced by Chance and Williams (1956, *loc. cit.*), which is concerned with the relative rates of change of the oxidation-reduction state of the respiratory carriers when respiration is increased by ADP, DNP and by oxygen.

QUANTITATIVE ASPECTS OF THE CONTROL OF OXYGEN UTILIZATION*

BRITTON CHANCE

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A RECENT review by Lardy (1956) summarizes many qualitative features of respiratory control, along lines suggested by Loomis (1949). Now, the properties of the reaction of adenosine diphosphate (ADP) and phosphate with the oxidative phosphorylation system can be studied quantitatively, and the suitability of these and other substances for key intermediates in metabolic control can be evaluated. We shall present, in this paper, a summary of the extent to which ADP and phosphate control electron transfer in mitochondria isolated from a wide range of tissues. Experimental studies of the effect of magnesium and calcium upon respiratory control and interpretations of their possible physiological function will also be given. The action of uncoupling agents upon respiratory control will be considered, as will the possibility that some uncoupling agents act selectively upon different sites of oxidative phosphorylation. Lastly, we shall present experimental evidence for the intramitochondrial substance responsible for respiratory control and discuss its possible nature. In an effort to determine which of the possible control substances determines the rate of respiration under physiological conditions, we have examined intact cells and tissues by optical methods that are responsive to metabolic control by substrates, coenzymes, and ADP or phosphate.

Measurement of respiratory control ratios

It is important to define respiratory control ratio as that of the respiratory rate in the presence of added ADP to the

* This research was supported in part by grants from the Office of Naval Reseach and the American Cancer Society.

rate obtained following its expenditure (Chance and M. Baltscheffsky, 1958). Loosely coupled mitochondria are much more easily detected by this criterion (see Fig. 2). Because the affinity of the mitochondria for ADP is so much higher than for phosphate (see below) and respiration therefore so much more easily started and stopped by small additions of

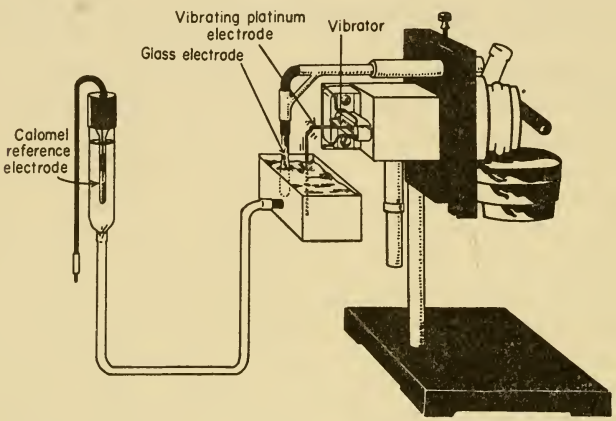

FIG. 1. Polarographic method for measuring oxygen utilization of mitochondrial suspensions employing a vibrating platinum microelectrode and a calomel reference cell connected to a salt bridge. The electrode is vibrated at 60 c.p.s. by the reed of a "Brown Converter." The electrical circuit converts the electrode current to an alternating voltage. (Expt. no. MD 33b)

ADP, we have, as a practical consideration, not used phosphate for a determination of respiratory control ratios.

Respiratory control ratios can be satisfactorily measured in the Warburg respirometer, as indicated by the work of Lardy and Wellman (1952). It has, however, been found more convenient to use polarographic methods employing either a stationary platinum electrode (Davies and Brink, 1942) with a rotating cup (G. R. Williams, unpublished) or a vibrating platinum microelectrode (Chance and Williams, 1955*a*). Of the two methods, the latter has a much more rapid response. An illustration of the arrangement of the platinum micro-

electrode and the reference electrode in conjunction with a spectrophotometric apparatus is given in Fig. 1. The glass-enclosed platinum tip is inserted well beneath the surface of the mitochondrial suspension and is vibrated up and down at an amplitude of about 1 mm. by the reed of a "Brown Converter" at 60 cycles per sec. Rapid vibration of the platinum tip is thereby obtained without undue disturbance of the solution. The electrode is polarized at $-0{\cdot}6$ v and reduces oxygen to either peroxide or water, depending upon the experimental conditions at the tip of the electrode. Thus, a current proportional to the oxygen concentration is obtained. This current passes through a salt bridge and calomel reference electrode and can be measured by any convenient electronic system. As we have made the circuit for the vibrating electrode, the current proportional to oxygen concentration is obtained as an alternating voltage which can be more readily amplified and recorded than a steady current.

Examples of respiratory control in mitochondria

In Fig. 2 (A-C) we see that some mitochondrial preparations which exhibit a low respiratory rate in the presence of substrate alone show a stimulation of respiration upon addition of ADP but a negligible slackening of respiration upon consumption of an amount of oxygen equivalent to the amount of added ADP. In Fig. 2A, the vibrating platinum electrode is inserted into a cuvette containing a suspension of heart sarcosomes in the usual saline medium for the assay of oxidative phosphorylation in liver mitochondria. In the presence of $2{\cdot}5$ μM α-ketoglutarate (α-Kg), the respiration rate is $0{\cdot}3$ μM O_2/sec. Shortly after the addition of 290 μM ADP, the respiration increases approximately threefold. However, instead of slackening after approximately 40 μM oxygen has been consumed, as would be expected from the P/O value for this type of preparation, the respiration continues practically unabated until the remaining oxygen (240 μM) is exhausted. In Fig. 2B we represent the response of another preparation of

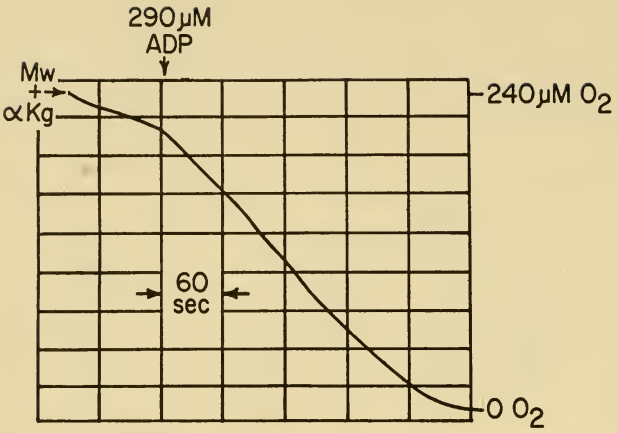

FIG. 2A. Illustration of the lack of respiratory control in heart sarcosomes suspended in saline medium. The addition of a small concentration of ADP accelerates respiration, but thereafter no appreciable deceleration occurs. (Expt. no. 305)

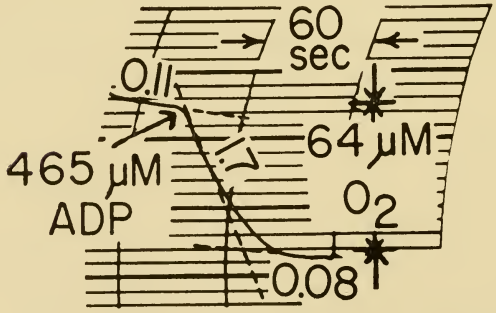

FIG. 2B. Control of respiration of a heart sarcosome preparation; sucrose - ethylene-diaminetetra-acetic acid medium, αKg as substrate. Note that the respiration rate decelerates abruptly when the added ADP has been expended. (Expt. no. 615)

heart mitochondria suspended in sucrose-ethylenediamine-tetra-acetic acid (EDTA) medium (Slater and Holton, 1954; Chance and M. Baltscheffsky, 1958). Again the mitochondria are pretreated with substrate and suspended in the aerated medium. Prior to the addition of ADP, the respiration rate is 0·11 μM O_2/sec.; following the addition of ADP, the respiration rate rises to 1·7 and, after 64 μM O_2 has been consumed, drops abruptly to 0·08. Thus, the ratio of the rate prior to

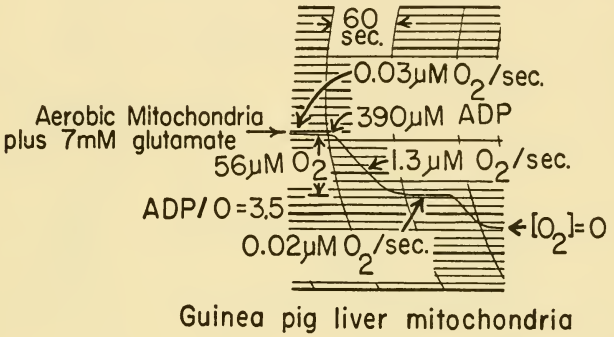

FIG. 2C. Control of respiration in a guinea-pig liver mitochondrial suspension. The respiratory control ratios here are among the highest observed for this experimental technique. (Expt. no. 372)

the addition of ADP and after the exhaustion of the added ADP exceeds 20 : 1. Fig. 2C gives the highest values of respiratory control that have been observed in our experiments; these were obtained with guinea-pig liver mitochondria. The substrate-treated mitochondria give a respiration rate of 0·03, which is increased to 1·3 by addition of 390 μM-ADP. After expenditure of the added ADP, the rate falls to 0·02. In this case, the ratio of the rates in the presence of ADP to those following the exhaustion of added ADP is apparently 65 : 1.

Experimental data on the optimal values of respiratory control obtained in mitochondria isolated from a variety of

sources are given in Table I. Since the measurement of respiratory control ratios in excess of 20 : 1 taxes the accuracy of the polarographic method used under these experimental conditions, we have, in this table, placed those ratios exceeding this value in parentheses. It is clear that respiratory

Table I

OPTIMAL VALUES OF RESPIRATORY CONTROL RATIOS IN ISOLATED MITOCHONDRIA

Source	*Medium*	*Substrate*	*Respiratory control ratio*	*Expt.*	*Reference*
Rat liver	Salts	Glutamate	5–15		Lardy and Wellman (1952)
Rat liver	Salts	β-hydroxy-butyrate	>15		Lardy (1956)
Rat liver	Salts	β-hydroxy-butyrate	6·6		Hoch and Lipmann (1954)
Rat liver	Salts	β-hydroxy-butyrate	>20 (38)	337	Chance and Williams (1955*a*)
Rat heart	Sucrose-EDTA	α-ketoglut-arate	>20 (21)	615	Chance and M. Baltscheffsky (1958)
Guinea pig liver	Salts	Glutamate	>20 (65)	372	Chance and Williams (1955*a*)
Guinea pig kidney	Tris-sucrose	Glutamate	17	644	
Turtle liver	Salts	Glutamate	8	641*	
Toad heart	Sucrose-EDTA	Succinate	5	815†	
Ascites tumour cells	Salts	Succinate	6	662	Chance and Hess (1959)

* Experiments in collaboration with Dr. F. Jöbsis.
† Experiments in collaboration with Dr. J. Ramirez.

control ratios of over 10 : 1 can be obtained from most tissues which have been carefully studied. Mitochondria prepared from cells in which the membrane is so resistant to rupture that damage to the mitochondrial membrane ensues may show respiratory control ratios of less than 10 : 1.

Most of the respiratory control ratios given in Table I were measured in the presence of a DPN-linked substrate

which shows a ratio several-fold greater than can be obtained with succinate. Thus, the values for toad heart muscle and ascites tumour cells, both of which were measured with succinate, would probably have been much larger had a DPN-linked substrate been used.

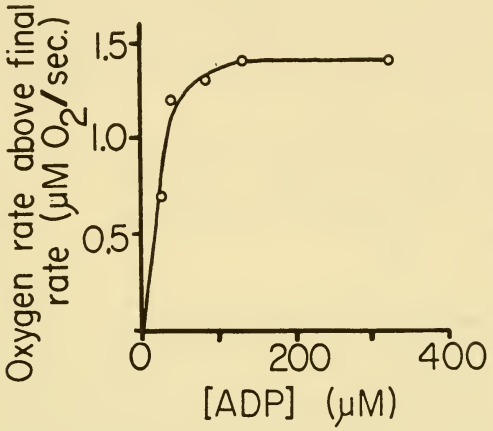

FIG. 3. The dependence of respiratory rate upon the added ADP concentration: guinea pig liver mitochondrial suspension with glutamate as substrate. Half-maximal acceleration is obtained in 20 μM ADP. (Expt. no. 379c) (From Chance and Williams, 1955*a*. Reproduced by permission of the Editors, *J. biol. Chem.*)

ADP and phosphate affinity of isolated mitochondria

By measurements of the stimulation of respiratory rate in response to additions of small concentrations of ADP, it is possible to obtain a measure of the ADP affinity of the respiratory chain. The vibrating platinum electrode is especially suitable for this type of experiment since its rapid response allows the measurement of respiratory rate a few seconds after adding a very low concentration of ADP. Fig. 3 indicates the effect of ADP concentration upon respiratory rate for a guinea-pig liver preparation with succinate as

substrate (Chance and Williams, 1955*a*). Approximately 25 μM ADP gives half-maximal respiratory stimulation. Two previous estimates of the ADP affinity of the respiratory chain have appeared in the work of Slater and Holton (1953). Their direct measurement gave a value of 7×10^{-7} which disagrees strongly with the results obtained here. Another value of 10^{-5}, obtained by a rather indirect estimation of the affinity of isolated mitochondria for endogenous ADP, is nevertheless within the range of values found here.

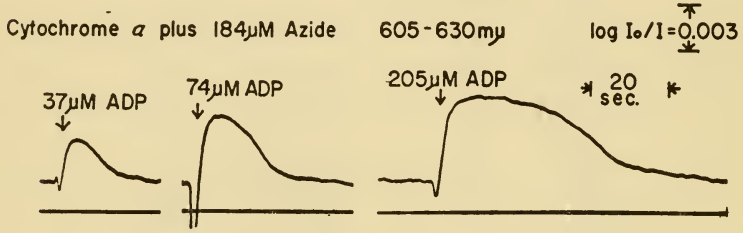

FIG. 4. Cyclic responses of cytochrome *a* of azide-treated guinea-pig liver mitochondria obtained in response to increasing concentrations of ADP. The separate additions of ADP are made to the same preparation after the effects of the preceding ones have gone to completion. (Expt. no. 388c) (From Chance and Williams, 1956*a*. Reproduced by permission of the Editors, *J. biol. Chem.*)

Spectroscopic measurement of the magnitude of effect of added ADP or phosphate upon the steady-state oxidation-reduction level of the respiratory carriers provides a much more sensitive method of determining the ADP affinity of the respiratory carriers themselves (Chance and Williams, 1955*b*). In Fig. 4, the mitochondria are diluted in a medium containing substrate and phosphate and are treated with a small concentration of azide so that a more distinctive response of cytochrome *a* to ADP addition can be obtained (Chance and Williams, 1956*a*). Thus, the addition of a small concentration of ADP (37 μM) causes a small cycle of reduction of cytochrome *a*, followed by its oxidation when the added ADP is expended.

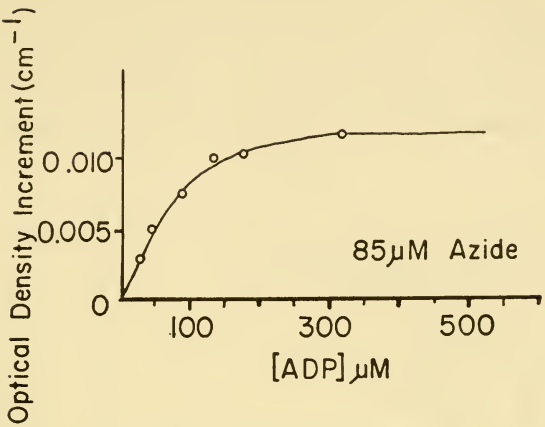

FIG. 5. The effect of ADP concentration upon the extent of reduction of cytochrome *a* in the steady state. The increasing concentrations of ADP cause increasing reduction of cytochrome *a* in the steady state, as indicated by Fig. 4. Guinea-pig liver mitochondria, succinate as substrate, 10° C. (Expt. no. 382b) (From, Chance and Williams, 1956*a*. Reproduced by permission of the Editors, *J. biol. Chem.*)

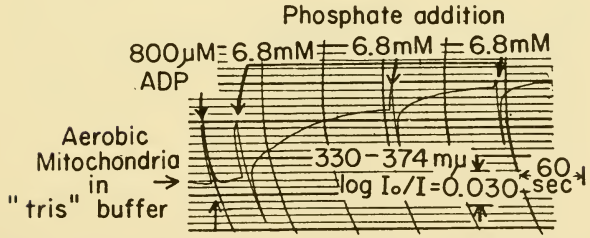

FIG. 6. Spectroscopic response of reduced pyridine nucleotide to addition of phosphate. Mitochondria are suspended in a phosphate-free medium and show no appreciable spectroscopic response to ADP addition. Successive additions of 6·8 mM phosphate causes oxidation of the steady state level of the reduced pyridine nucleotide. (Expt. no. 299b–19)

In the other two tests, the larger amounts of ADP cause greater reduction of cytochrome in the steady state and a longer duration of the cycle corresponding to the longer time required to expend the added ADP. By plotting the amplitude of these cycles against the added ADP concentration, we obtain a saturation curve, indicated in Fig. 5. Half-maximal

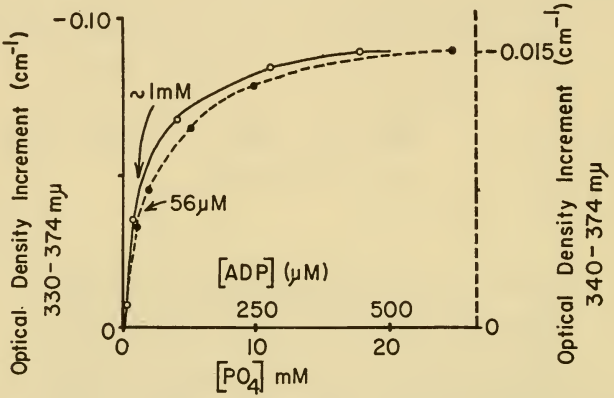

FIG. 7. Comparison of the effects of phosphate and ADP upon the steady state oxidation-reduction level of intramitochondrial reduced pyridine nucleotide. Addition of one of these substances to a mitochondrial suspension containing the other causes an oxidation of the reduced pyridine nucleotide. The relationship between the ADP and phosphate concentrations and the extent of oxidation of the pyridine nucleotide is indicated by the graph. The concentrations giving half-maximal effect are also indicated. (Expt. no. 299b, 463b) [From Chance and Connelly, 1957. Reproduced by permission of the Editors, *Nature (Lond.)*.]

effect is caused by 60 μM ADP under these conditions. A similar experiment may be carried out with phosphate, provided the substrate- and ADP-treated mitochondria are suspended in a phosphate-free medium. As illustrated by Fig. 6, such mitochondria show a negligible response to the addition of an excess of ADP. The respiratory carrier (in this case, reduced pyridine nucleotide) shows a slow increase of oxidation in response to the addition of 6·8 mM phosphate,

and further oxidation in response to further additions of phosphate. It would, therefore, appear that phosphate affinity is considerably lower than ADP affinity. The results of these studies are summarized and compared in Fig. 7 (Chance and Connelly, 1957), where pyridine nucleotide shows a half-maximal response at 56 μM ADP and 1 mM phosphate. These constants determine the response of the mitochondria of the intact cell to metabolic changes involving concentration changes of ADP and phosphate. ADP is the more effective: the mitochondria respond to much smaller changes of its concentration, and a low concentration of ADP will be more rapidly expended and brought down to a low final concentration. The response to phosphate will not occur until a high concentration has accumulated, and will be prolonged due to both the large amount and the slow utilization of concentrations below 1 mM. An increment of phosphate that would stimulate half-maximal respiration would cause an interval of respiration 20 times greater than one stimulated by an increase of ADP concentration. In other words, the response to phosphate will be sluggish, and the response to ADP will be rapid.

Effect of magnesium

It has been recognized for some time that maximal phosphorylation efficiency of isolated mitochondria may require added magnesium (Cross *et al.*, 1949). More recently, however, it has been shown that mitochondria may initially contain sufficient magnesium to permit a high phosphorylation efficiency, though one of short duration (Baltscheffsky, 1956). It has also been found that either endogenous or added magnesium also increases the respiratory control ratio (Chance, 1956). An example of the effect of magnesium upon the respiratory control of rat liver mitochondria is given in Fig. 8. In this case, mitochondria suspended in a sucrose-EDTA-phosphate medium show respiratory stimulation upon addition of succinate. After about a minute, 6 mM magnesium causes an immediate reduction of respiratory rate of about

sevenfold (Chance, 1956). Proof that the deceleration of respiration is due to the restitution of respiratory control and not to an unknown inhibition of electron transfer is demonstrated by the fact that ADP addition gives an even more rapid respiratory rate (a 14-fold increase).

To determine the magnesium concentration needed for

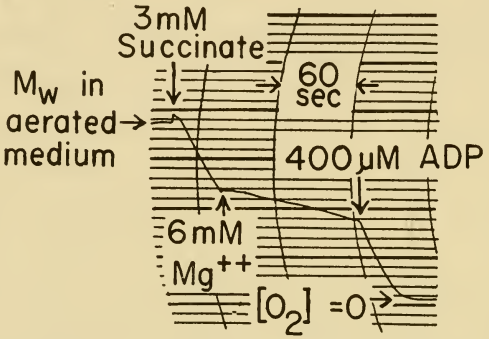

FIG. 8. An illustration of the increased respiratory control occasioned by the addition of magnesium to rat liver mitochondria suspended in sucrose-EDTA-phosphate medium. M_W = mitochondrial suspension. (Expt. no. 474b) (From Chance, 1956. Reproduced by permission of the Editors, *Proc. III int. Congr. Biochem.*)

restoration of half-maximal respiratory control in the suspension described above, we have plotted, in Fig. 9, the respiration rate for a number of magnesium concentrations. As shown in Fig. 8, the addition of 6 mM magnesium causes a maximal deceleration of respiration of more than sevenfold. Half-maximal effect is obtained with roughly 0·5 mM magnesium. Thus, inhibition of respiratory activity can be obtained by additions of low concentrations of magnesium to the mitochondria. Cyclic responses of respiratory activity have not been demonstrated for increases and decreases of magnesium concentration, as they have for ADP (Fig. 4). However, it is reasonable to assume that such responses would

FIG. 9. Magnesium concentration required for activation of the respiratory control mechanism. Experimental conditions similar to those of Fig. 8: sucrose-EDTA-phosphate medium, succinate as substrate. (Expt. no. 470d)

also occur in mitochondria subjected to cyclic changes in magnesium concentration.

Effect of calcium

Calcium has for some time been recognized as an uncoupling agent for mitochondria (Potter, 1947; Lehninger, 1949; Slater and Cleland, 1953). Furthermore, its addition causes loss of respiratory control or "decontrol", and the mechanism of its action involves several novel facets that merit discussion (Chance, 1956), especially in connexion with the effects of ADP and magnesium. Fig. 10 is a polarographic recording of the respiratory activity of a guinea-pig liver mitochondrial suspension treated with glutamate as substrate. Addition of 135 μM ADP causes an acceleration of respiration to a rate

of 1 μM O_2/sec. After the added ADP is expended, the respiratory rate falls by a factor of about 25. Addition of 385 μM calcium accelerates the respiration to 1·7 μM O_2/sec. It should be noted that calcium gives a higher rate than that obtained with ADP alone. After approximately 20 seconds,

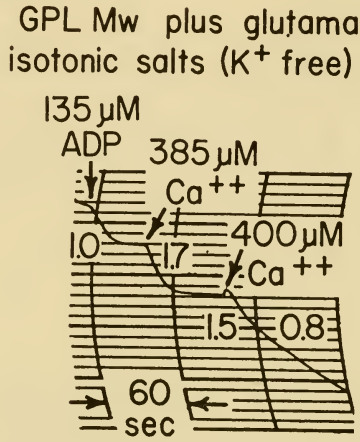

FIG. 10. Comparison of the effects of ADP and calcium upon respiratory rate in guinea-pig liver mitochondrial suspensions (GPL M_W). The vibrating platinum electrode (see Fig. 1) is used to record the respiratory rates as a function of time. Starting at the left-hand edge of the figure, decreasing oxygen concentrations are recorded in response to oxygen utilization caused by addition of ADP and calcium (Expt. no. 466c)

the respiration comes spontaneously to a halt, the control ratio in this case being approximately 40. A further addition of 400 μM calcium causes an acceleration of respiratory rate to 1·5, but at the end of 20 seconds, instead of falling to zero, it proceeds at 0·8 for the remainder of the experiment; respiratory control is never regained.

The calcium concentration for maximal stimulation of respiratory activity depends upon the magnesium concentra-

tion, as indicated in Fig. 11. In carrying out these experiments, the respiration rates were measured immediately after adding calcium to avoid the diminution of respiratory activity which characteristically occurs some seconds later.

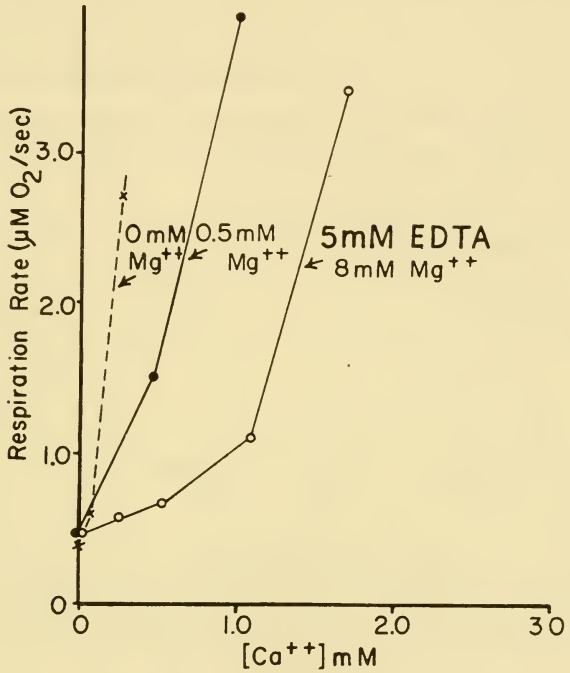

FIG. 11. Relationship between added magnesium and calcium concentrations required for activation of respiration. Experimental conditions similar to those of Fig. 10. (Expt. no. 473b)

In the absence of added magnesium (a sucrose-phosphate medium), a very small concentration of calcium gives half-maximal stimulation; approximately 80 μM was adequate in this experiment with succinate as substrate. This calcium concentration is only slightly higher than the ADP concentration required for half-maximal stimulation of respiration. If the reaction is carried out in the usual

sucrose-EDTA-phosphate medium containing the usual amount of magnesium, the mitochondria are quite resistant to calcium. Maximal respiration would therefore appear to require 2 mM calcium, half-maximal effects being obtained at about 0·5 mM calcium.

Respiratory control and oxidative phosphorylation

Experimental data show that loss of respiratory control is not necessarily accompanied by a significant decrease of oxidative phosphorylation efficiency. Even the earliest studies of phosphorylation efficiency showed that reasonably high P/O values could be obtained in preparations demonstrating no measureable change of respiratory rate in the presence or absence of phosphate acceptor. Although it is also generally observed that mitochondrial preparations exhibiting a high degree of respiratory control (Lardy and Wellman, 1952) show a high phosphorylation efficiency, recent experiments indicate that the two phenomena are readily dissociable in intact mitochondria. Hoch and Lipmann (1954) have shown that the effect of phosphate acceptor is almost abolished although the P/O ratio of the same preparation does not decrease. In a preparation whose respiratory control ratio, in the absence of dinitrophenol (DNP), is approximately 7 : 1, 10^{-5} DNP causes only a 23 per cent decrease of the P/O ratio and a 72 per cent drop in the acceptor effect. Chance and Williams (1955*a*) have noted similar phenomena using butyl 3:5-di-iodo-4-hydroxybenzoate (DIB). For a preparation which in the absence of the uncoupling agent, shows a respiratory control ratio of 7 : 1 and a P/O value of 3·0, the addition of the uncoupling agent causes the respiratory control ratio to fall to 1·41 while the P/O value remains essentially unchanged (3·1). Further experiments on the magnesium-deficient system studied by H. Baltscheffsky (1957) show that lack of magnesium can cause a similar loss of respiratory control under conditions which do not significantly affect the P/O value. It would appear that the "R factor" discussed by

Lehninger (this symposium, p. 140) is comparable in its effect with low concentrations of the substance described here.

These data make it evident that respiratory control can be diminished by a number of compounds without necessarily interfering with the thermodynamic efficiency of the cell in its energy conservation reaction.

Effect of uncoupling agents

Fig. 12 illustrates the effect of dicoumarol upon the respiratory rate of rat liver mitochondria with β-hydroxybutyrate as

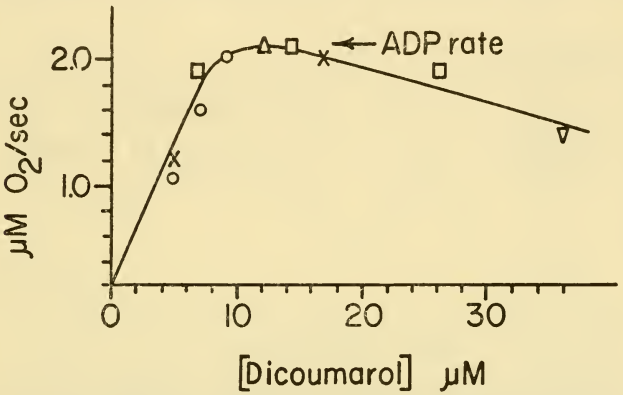

FIG. 12. Effect of dicoumarol upon the respiratory rate of a rat liver mitochondrial suspension; β-hydroxybutyrate as substrate. The respiratory rate obtained with excess ADP in an independent experiment is indicated by the arrow. (Expt. no. 468c)

substrate. The respiratory rate increases with dicoumarol concentration, rises to a maximum value, and then declines at higher dicoumarol concentrations. The concentration required for half-maximal activation of respiration is 4 μM. It is significant that the rate obtainable in the presence of ADP and in the absence of dicoumarol is the same (see arrow, Fig. 12).

Since it has been postulated that dicoumarol has a specific effect upon electron transfer between reduced pyridine nucleotide and cytochrome *b* that differs from its effect upon

the other components (Martius, 1956), we have endeavoured to learn whether dicoumarol concentrations which cause loss of respiratory control equally affect the respiratory carriers. We have, therefore, carried out a dicoumarol titration of the steady-state oxidation-reduction level of cytochrome *c* (Fig. 13) in the presence of azide to increase the spectroscopic effects. The same dicoumarol concentration (4 μM) is required

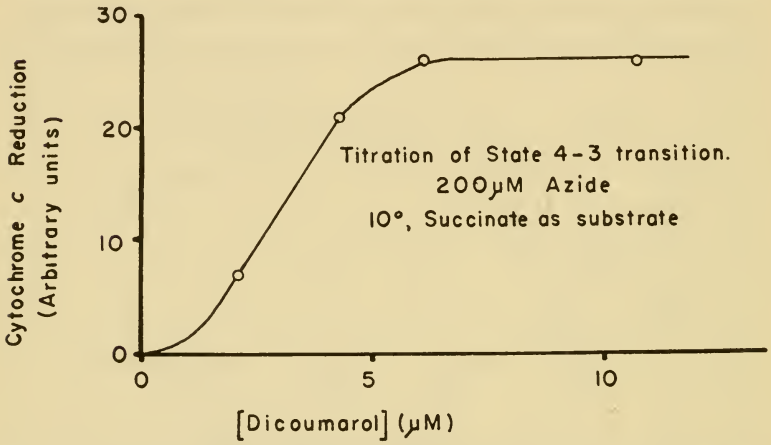

FIG. 13. Titration of the steady state oxidation-reduction level of cytochrome *c* with the uncoupling agent, dicoumarol. Mitochondria pretreated with azide, succinate as substrate. Increasing cytochrome reduction is caused by increasing dicoumarol concentration. (Expt. no. 529)

for half-maximal interaction with the cytochrome *c* component as is required for half-maximal activation of respiration. There are, however, slight differences in the experimental conditions that might make this close agreement fortuitous: the data of Fig. 13 were obtained at lower temperatures and with succinate as substrate. Nevertheless, we can find no significant discrepancy between the response of the carriers and the response of the whole chain to dicoumarol that would lead us to believe that one site of phosphorylation can be uncoupled independently of the others by dicoumarol. Further evidence for the similarity of the site specificity of

dicoumarol and ADP has been mentioned previously (Chance, 1956), and is repeated here (Fig. 14). Here we compare the absorption bands of the cytochromes which disappear upon addition of ADP with those which disappear upon addition of dicoumarol. If, for example, there were a selective effect of dicoumarol, then one would expect differences in the responses to these agents. Treatment of the mitochondria with

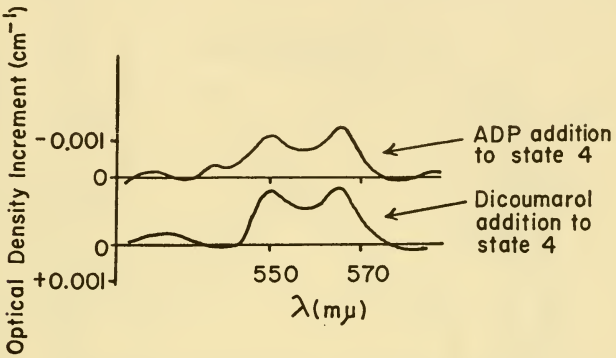

FIG. 14. Spectroscopic changes caused by addition of ADP, upper trace, compared with those caused by dicoumarol, lower trace. Both traces show an oxidation of cytochromes *c* and *b*. The data are plotted so that a decrease of absorption corresponds to an upward deflexion on the traces. Rat liver mitochondria, 400 μM ADP, 14 μM dicoumarol, β-hydroxybutyrate as substrate. (Expt no 468d–6) (From Chance, 1956. Reproduced by permission of the Editors, *Proc. III int. Congr. Biochem.*)

ADP causes absorption bands due to cytochromes $c + c_1$ (551 mμ) and *b* (562 mμ) to disappear, as indicated by the double-humped peak in the difference spectrum. Addition of dicoumarol affects the absorption bands to the same relative extent.

An interesting feature of Fig. 14 is that the absorbancy changes caused by dicoumarol are slightly larger than those caused by ADP, which appears to be inconsistent with the nearly identical respiratory stimulation caused by these two substances. In order to determine whether this is due to

dicoumarol or whether it can be observed with other uncoupling agents, we have made studies of the effect of DIB*:

Fig. 15A indicates the results of a series of experiments in which the effect of DIB upon the steady state of cytochrome *b* is compared with its effect upon respiration with glutamate as substrate. The respiratory activity rises to a maximum at 4 μM DIB, and to a half-maximal level at 2 μM DIB. The

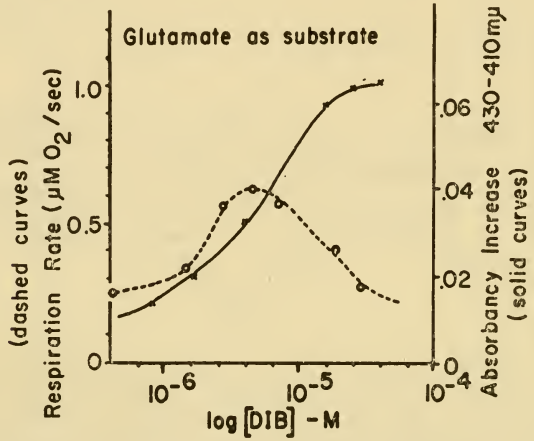

FIG. 15A. A comparison of the spectroscopic and respiratory effects of DIB. Guinea-pig kidney mitochondria, glutamate as substrate. (Experiments carried out in collaboration with Dr. G. Hollunger.) (Expt. no. 658a)

change of steady state level of cytochrome *b* shows a significantly different response: absorbancy increase reaches a plateau only when inhibition is maximal. It is apparent that two overlapping spectroscopic responses to DIB are involved. One gives a spectroscopic change of magnitude similar to that caused by ADP at concentrations of the uncoupling agent which give maximal respiratory rate. The second is a further increase in the oxidation of cytochrome *b*, which continues until inhibition is complete. These two responses

* Kindly donated by Dr. H. Lardy.

apparently overlap to such an extent that no inflexion of the spectrophotometric effect is observed at DIB concentrations corresponding to maximal respiratory activity.

With most uncoupling agents, it is difficult to observe their effect upon the steady-state level of reduced pyridine nucleotide because of their absorption in this region. With DIB,

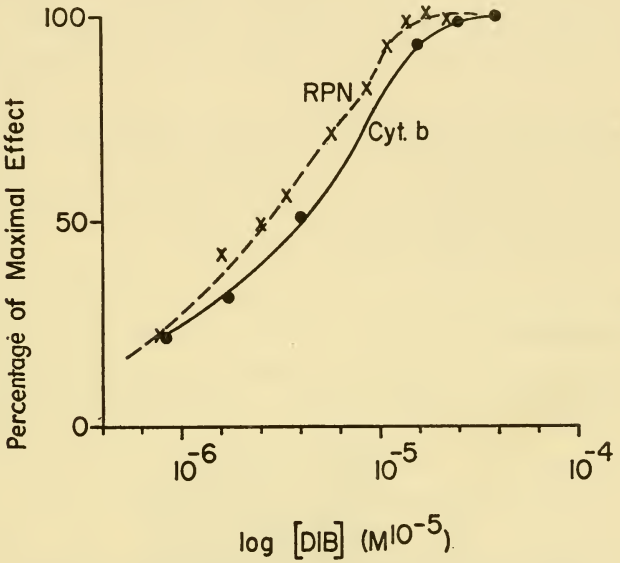

FIG. 15B. Comparison of the spectroscopic effects of DIB on reduced pyridine nucleotide and cytochrome *b* of guinea-pig kidney mitochondria; glutamate as substrate. (Experiment carried out in collaboration with Dr. G. Hollunger.) (Expt. no. 658a)

however, measurements of reduced pyridine nucleotide at 350 mμ with respect to 374 mμ are relatively insensitive to its absorption. It has, therefore, been possible to make a titration of the effect of DIB upon reduced pyridine nucleotide. Oxidation of both reduced pyridine nucleotide and cytochrome *b* is indicated in the graph of Fig. 15B in which the results are plotted as percentages of the maximal effect. It is seen that the curves are very similar in shape and that half-maximal

effects are obtained at 1·6 and 2·5 μM DIB for reduced pyridine nucleotide and cytochrome *b*, respectively, with glutamate as substrate. These results indicate that the uncoupling response of the pyridine nucleotide is very similar to that of cytochrome *b*. The fact that pyridine nucleotide becomes further oxidized in the presence of excess uncoupling agents indicates that the inhibition of respiratory activity with excess concentrations of the uncoupling agents is due to interference with the reduction of pyridine nucleotide. This is a point of considerable interest, since the isolated dehydrogenases are not sensitive to uncoupling agents. This result calls our attention to the possibility that the reduction of pyridine nucleotide in mitochondria may be driven by high-energy intermediates, not only with succinate as substrate, but with other substrates as well.

Respiratory control for the substrate level in mitochondria

The affinity of mitochondria for most substrates is so low that it is difficult to demonstrate control of electron transfer

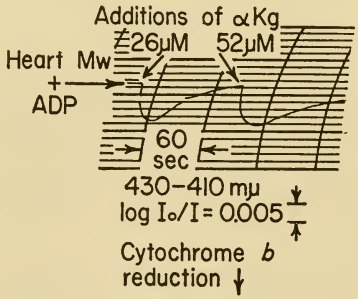

FIG. 16. Cyclic response of cytochrome *b* of mitochondrial suspension to increases and decreases of substrate concentration. Rat heart mitochondria, pretreated with ADP; αK as substrate (Expt. no. 618).

in response to small additions of substrate; amounts of substrate sufficient to elicit maximum activity require a very

long interval for their consumption. α-Kg is one substrate with which it is possible to demonstrate the cyclic control of respiration, and a typical record is presented in Fig. 16. If the mitochondria are pretreated with ADP and phosphate, addition of α-Kg will cause reduction of cytochrome *b* which proceeds to a maximum value, remains in a steady state, and declines as the added substrate is expended. The cycle can be repeated by a further addition of α-Kg. With this particular substrate, the control is almost as effective as that obtained with ADP.

Nature of intramitochondrial pyridine nucleotide

Various experiments indicate that the inhibitory substance responsible for respiratory control is present in the mitochondria in amounts closely corresponding to their pyridine nucleotide content. Among a number of data in favour of this conclusion, two are especially clear: (*a*) the titration of intramitochondrial reduced pyridine nucleotide by added ADP in a 1 : 1 stoichiometric reaction (Chance, 1958), and (*b*) the rapid phosphorylation of added ADP to ATP (adenosine triphosphate) in amounts stoichiometrically related to the intramitochondrial pyridine nucleotide (Schachinger, Eisenhardt and Chance, 1958). Some recent experiments give evidence that intramitochondrial pyridine nucleotide is a bound form of pyridine nucleotide that is extremely labile in the presence of substances which modify the mitochondrial structure, as is indicated by the fluorescent light emitted by mitochondria when they are illuminated at wavelengths between 340 and 366 mμ (Chance and Conrad, 1958; Chance and H. Baltscheffsky, 1958). Fig. 17 shows that the maximum for the emission spectrum of mitochondrial fluorescence occurs at 443 mμ, in almost exact agreement with the maximum for light emission for pyridine nucleotide bound to yeast alcohol dehydrogenase (Duysens and Kronenberg, 1957). Many data indicate that this fluorescent material is intramitochondrial reduced pyridine nucleotide. Perhaps the most convincing are those based upon the close correspondence of

its kinetics to those of the 340-mμ absorption band in response to a variety of chemical treatments. Attempts have been made to see whether a bound form could be obtained in solution, and experiments to date indicate that even slight

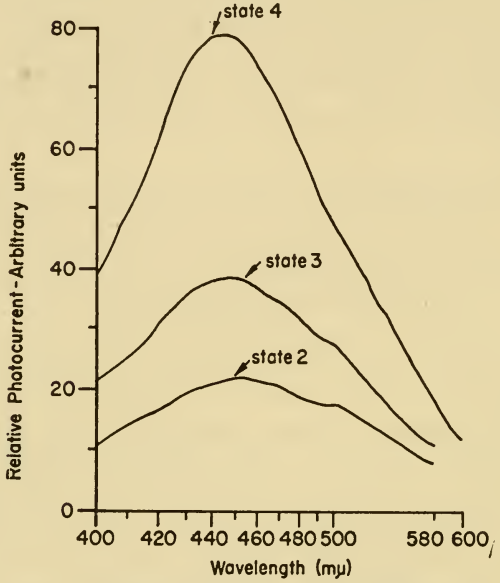

FIG. 17. Emission spectrum of fluorescence of mitochondrial suspension excited at 353 mμ. Rat liver mitochondria, β-hydroxybutyrate as substrate. Emission spectrum corrected for transmission of monochromator and sensitivity of photo surface (Expt. no. 777) (From Chance and H. Baltscheffsky, 1958. Reproduced by permission of the Editors, *J. biol. Chem.*)

damage to the mitochondrial membrane causes disappearance of the fluorescence.

The existence of this compound, together with the very high degree of reduction of intramitochondrial pyridine nucleotide in the absence of ADP or phosphate, gives strong support to the hypothesis that the high respiratory control ratio, characteristic of tightly coupled mitochondria utilizing

DPN-linked substrates, is due to the unreactivity of such a bound form of pyridine nucleotide towards oxidation by flavoprotein unless the following transfer reactions occur (Chance and Williams, 1956*b*). Here the two steps of the phosphorylation reaction are written as suggested by the data of Plaut (1957):

$$\text{DPNH} \sim \text{I} + \text{X} \rightleftharpoons \text{DPNH} + \text{X} \sim \text{I} \quad (1)$$

$$\text{X} \sim \text{I} + \text{P} \rightleftharpoons \text{X} \sim \text{P} \quad (2)$$

$$\text{X} \sim \text{P} + \text{ADP} \sim \text{ATP} \quad (3)$$

Application to metabolic control problems

Two factors need to be considered in connexion with the rôle of the mitochondria in control of metabolism: whether control of respiratory metabolism is exercised by the substrate level or through the phosphorylation process.

Control of respiration at the substrate level. Baker's yeast suspended in phosphate buffer and aerated for 18 hours has a very low respiratory activity. Under such circumstances, the initiation of metabolism occurs directly upon the reduction of intramitochondrial pyridine nucleotide and cytochrome, since an excess of ADP has accumulated in the mitochondria. An example of the initiation of respiration in a starved yeast cell suspension is given by Fig. 18. The initiation of respiration caused by the addition of 34 mM ethanol has been recorded polarographically by means of the platinum electrode, and spectrophotometrically by the double-beam spectrophotometer at wavelengths appropriate for pyridine nucleotide and cytochrome *b*. In the right-hand portion of the figure, the rapid reduction of cytoplasmic and mitochondrial pyridine nucleotide through alcohol dehydrogenase activity is indicated by the downward sweep of the trace, corresponding to an increasing absorption at 340 mμ with respect to 374 mμ. The increased slope of the platinum microelectrode trace indicates a higher respiratory activity. In the left-hand portion of the figure, at wavelengths appropriate to cytochrome *b* (428–405 mμ), a similar, although slightly less abrupt,

reduction of cytochrome *b* is indicated by the downward sweep of the trace. Here, again, the platinum microelectrode indicates the rapid initiation of respiration.

Initiation of respiratory activity upon glucose addition is also a rapid but somewhat more complicated reaction (Chance, 1954, 1955). An interesting result of the glucose experiments is that pyridine nucleotide reduction at the level of glyceraldehyde-3-phosphate dehydrogenase can activate respiration. The rapid reduction of mitochondrial cytochrome by cyto-

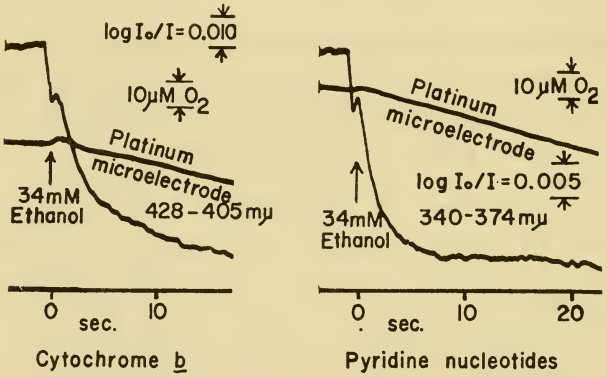

FIG. 18. A comparison of the kinetics of reduction of pyridine nucleotide and cytochrome *b* of starved yeast cells caused by the addition of ethanol. (Expt. no. 181)

plasmic dehydrogenase may result from the permeability of yeast mitochondria to reduced pyridine nucleotide formed in the cytoplasm. On the other hand, there may be a much closer organization of glycolytic and mitochondrial enzymes within the cell than can be demonstrated in cell extracts.

In flight muscle of the housefly, it is suggested that the substrate level may control the respiratory activity (Chance and Sacktor, 1958).

Metabolic control through the phosphorylation system. The more subtle aspects of metabolic regulations are not usually revealed by the transition from the "starved" to the "fed" state, but instead require the study of transition between

more delicately balanced levels of metabolic activity. It is, in fact, possible not only to demonstrate control of respiratory metabolism, but also to show that this is due to limitation of intracellular ADP concentration. Yeast cells are appropriate for this type of study. Fig. 19 represents, in its initial phases, a repetition of the experiment of Fig. 18 in which aerated,

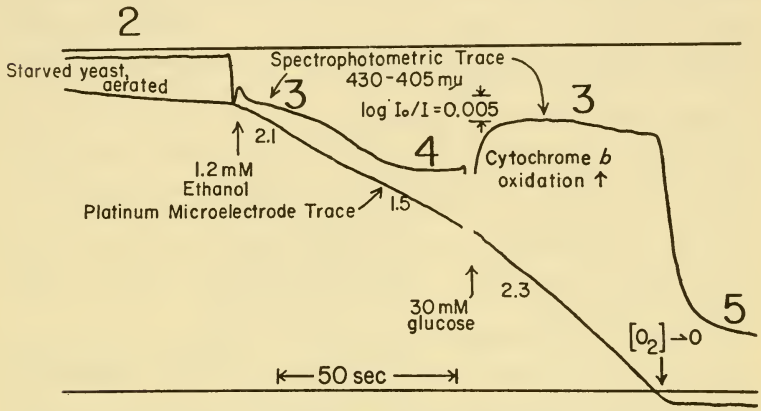

FIG. 19. Identification of ADP as a rate-controlling intermediate in the respiratory metabolism of baker's yeast cells. Combined spectrophotometric and polarographic recordings of cytochrome *b* reduction and respiratory activity. The large numbers refer to the metabolic state of the yeast (2 starved, 3 active and phosphorylating, 4 inactive due to lack of ADP or phosphate, 5 anaerobic). The small numbers below the respiratory trace indicate oxygen uptake rates in μM/sec. (Expt. no. 493b–14) (From Chance and Williams, 1956*b*. Reproduced by permission of the Editors. *Advanc. Enzymol.*)

starved yeast is treated with ethanol. In this case, however, a lower concentration of ethanol is used. As in the previous experiment, a rapid reduction of cytochrome *b* is indicated by the downward deflexion of the spectrophotometric trace represented by the recording of absorbancy changes at 430 mμ with respect to 405 mμ. Respiration starts rapidly and achieves a steady-state rate of 2·1 μM O_2/sec., as indicated on the trace. The mitochondria are now in an active state, respiring rapidly with an excess of both substrate and intracellular ADP. They are therefore in state 3. About a minute

after addition of ethanol, there is a further downward sweep of the spectrophotometric trace, indicating further reduction of cytochrome *b*. The increased absorbancy at 430 mμ is suggestive of a transition in the mitochondria of state 3 to state 4. There is a corresponding decrease of respiratory rate from 2·1 to 1·5 μM O_2/sec. This is not a very large decrease, and is in agreement with other considerations that indicate that the yeast cell may not represent a "tightly coupled" system. The possibility that respiration was slackened due to lack of substrate is disproved by the fact that cytochrome becomes more reduced, indicating, if anything, an increased substrate concentration. In view of the foregoing discussions, this effect could be attributed to a number of control substances: a decrease of ADP or phosphate concentration, an increase of magnesium concentration, or a decrease of any physiological uncoupling substance such as calcium, mitochrome, lipid, etc.

Evidence for the type of substance exerting respiratory control over the mitochondria of the yeast cell is afforded by both the spectroscopic and the respiratory responses of the cell suspension to glucose addition (Fig. 19). The rapid upward deflexion of the spectrophotometric trace to the level obtained a few seconds after adding ethanol indicates that whatever control substance was depleted a minute after the addition of ethanol has been restored. This is supported by the fact that the respiration increases to a value somewhat above that obtained shortly after ethanol addition (2·3 μM O_2/sec.).

It is clear, from the known pathway of glucose metabolism of the yeast cell and from the great rapidity with which its respiration is accelerated upon glucose addition, that ADP produced in glucose phosphorylation has reactivated the respiratory chain. An increase of substrate concentration is eliminated by the spectroscopic response, indicating an oxidation of cytochrome *b*. This type of spectroscopic change could only be caused by a decrease of substrate concentration. An increase of phosphate concentration is unlikely, since the primary reaction involves the formation of ADP, phosphate

remaining attached to glucose. Thus, it is apparent that sufficient phosphate was available inside the cell to permit the rapid restarting of respiration solely in response to an increase of intracellular ADP concentration. The spectroscopic and respiratory changes could not be attributed to the transport of magnesium or calcium into the cell at the moment glucose was added because the yeast was suspended in a calcium-free buffer solution.

Similar experiments show respiratory control to be a factor

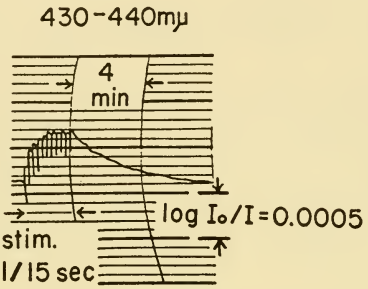

FIG. 20. Response of cytochrome *b* component of frog's sartorious muscle to a series of twitches. Each twitch is indicated by the interruptions of the trace. Oxidation of cytochrome *b* is indicated by an upward deflexion. (Expt. no. W–15) (By courtesy of Dr. A. Weber.)

in the steady state metabolism in other tissues (ascites tumour cells) (Chance and Hess, 1956). The glucose experiment is perhaps the most useful test for ADP-limited control. In muscular contraction, control due to ADP or phosphate can be demonstrated by the spectroscopic response of the cytochromes. An example of this is afforded by the oxidation of cytochrome *b* of excised frog sartorius muscle caused by a series of single contractions (Weber, 1957), which gives a maximal steady state response (Fig. 20). At the same time, utilization of the accumulated ADP or phosphate occurs and

the steady state characteristic of the resting muscle is re-established.

Discussion and summary

A desirable characteristic of a respiratory control chemical is that it be capable of triggering the system on and off by small changes in its concentration. When this is the case, the system may be set into full activity with a small increase of concentration and can subside to the resting state with a small decrease of concentration. Such trigger-control characteristics have the advantage of preventing the accumulation of large amounts of control substance prior to the complete activation of the system. Poor candidates for this type of control in mitochondria are phosphate, magnesium and, in most cases, substrate; the system has sufficiently low affinity for these substances that it cannot be triggered with low concentrations. On the other hand, both ADP and calcium can trigger the system at low concentrations and can stimulate it to maximal activity in response to a small amount of the control substance.

High affinity for the control substance allows simultaneous control of a number of processes. For example, the relatively higher ADP affinity of mitochondria compared to the phosphoglucerate kinase gives the mitochondria the possibility of controlling the activity of the Embden-Myerhof pathway. On the other hand, the affinity of the mitochondria for phosphate is considerably lower than for ADP, and thus phosphate cannot compete as effectively with phosphorylation steps in glycolysis which involve this substance. Metabolic control in terms of the substrate level may have the disadvantage of low affinity. A further disadvantage is that substrate addition to any part of an enzyme sequence may not activate the whole system simultaneously. Addition of substrate would activate the system in a sequence starting with those enzymes nearest the substrate, since the concentration of various intermediates needs to be built up; the converse is true for the expenditure of substrate. In this respect, the ADP control of electron

transfer in the mitochondria has a noteworthy advantage, for all sites of oxidative phosphorylation are affected simultaneously by ADP addition.

In summary, ADP appears to be the most favourable candidate for delicate regulation of metabolic processes.

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DISCUSSION

Racker: Prof. Chance, I noticed that all your experiments were carried out at a very low level of total adenine nucleotide concentration, i.e. 3×10^{-4} M, which is far below the intracellular concentration. I wonder whether the very sharp respiratory control which you have observed could perhaps be an artifact due to the low concentration of adenine nucleotide which you use. If one measures the ATPase activity of mitochondria in the presence of an ATP-regenerating system with phospho*enol*pyruvate and pyruvate kinase which remove inhibitory ADP, one can observe very much higher levels of ATPase activity than with the conventional method. We believe that an ATPase activity may well be functional during oxidative phosphorylation provided that the ATP is not channelled into some more useful purposes. We find that when we measure respiration in liver mitochondria we observe little or no oxygen uptake in the absence of ATP, but on addition of ATP even without any acceptor we observe considerable uptake of oxygen. Of course, there is further stimulation of respiration on the addition of a phosphate acceptor such as glucose and hexokinase or on the addition of ATPase. I wonder whether the respiratory control in the cell is really as complete as you have proposed. Sir Hans has emphasized many times that the Krebs cycle (or citric acid cycle, as he calls it) is not only providing energy by oxidative phosphorylation but it participates also in synthetic processes such as the formation of glutamic acid. We could visualize here a release mechanism due to ATPase which allows for a certain amount of substrate to be getting through the Krebs cycle, even under conditions of overproduction of ATP.

Chance: I presented the yeast experiment because it showed that, in cells of this type, we did not get more than a 50 per cent decrease in respiratory rate, at a time when we observed a marked drop of intracellular ADP. If we use ascites tumour cells, they show a much more dramatic drop (over tenfold, see p. 119).

Racker: But even in the ascites cells, the complete standstill of respiration after addition of glucose is very brief; at least in our experiments it lasts only two or three minutes and then respiration is resumed. In other words, the complete inhibition of respiration is only short-lived.

Chance: This blockage affords an excellent way of studying the mechanism of control of oxidative metabolism. On a quantitative basis, the Q_{O_2} at 26° in the blocked state is 2–6 and is stable for over 10 minutes. In the rapid phase the Q_{O_2} is 20–30. Thus, a fairly active metabolism for this type of cell proceeds in the "blocked" state.

Hess: In order to illustrate the significance of this blocking of oxidative metabolism I shall give some quantitative chemical data, first on the critical stationary ADP concentration of resting mitochondria in the blocked state 4 and, secondly, as applied to intact cells, on the glucose-oxygen stoichiometry in ascites tumour cells. The ADP and ATP concentration in resting mitochondria (state 4) was measured after deproteinization, by means of enzymic tests, ADP by using the pyruvate

kinase-lactic dehydrogenase system, ATP by using the phosphoglycerol kinase-glyceraldehyde phosphate dehydrogenase system. Our results are given in Table I. For each experiment the concentration of ATP

Table I (HESS)

ATP/ADP RATIO IN ISOLATED LIVER MITOCHONDRIA (STATE 4)

	Concentration μM/ml.				
Expt. No.	*ATP*	*ADP*	*ATP + ADP*	*ATP/ADP*	*ADP added*
1	0·105	0·019	0·124	5·5	*endogenous*
2	0·430	0·028	0·458	15·4	0·4
3	0·380	0·038	0·418	10·0	0·4
4	0·380	0·057	0·437	7·8	0·4
5	0·370	0·047	0·415	8·0	0·4

Conditions: phosphate buffer, pH 7·4 (0·01 M) β-hydroxybutyrate 10 μM, Mg^{2+} 50 μM, mitochondria: 8·8 mg. biuret value/ml.

and ADP, the sum of both and the ratio is given. Experiment 1 shows the results giving the endogenous concentration without addition of external ADP. Here the steady state concentration of ADP is very low and well within the range we would expect from the titration data for the ADP affinity (K_m) for the respiratory chain below 56 μM which have been given by Prof. Chance. In experiments 2–5, ADP was added from outside to the mitochondria, which were deproteinized and analysed after state 4 was reached according to the oxygen kinetics recorded with a platinum electrode. Here, again, the steady state concentration of ADP is very low and within the expected range, but not as low as in experiment 1. This small increase might be due to methodological factors. The time required for deproteinization and analytical treatment of the samples is enough to allow some ATP to be broken down to ADP and to spoil the analysis. However, the values for the ATP/ADP ratio illustrate quite well the high efficiency of the mitochondria in keeping the theoretically expected steady state value of ADP.

In order to demonstrate a pathological, abnormal efficiency of mitochondria, it is useful to influence the ATP/ADP ratio by means of triiodothyronine or thyroxine, the uncoupling action of which was shown in 1951 (Martius, C., and Hess, B. (1951). *Arch. Biochem.*, **33**, 486). The influence of tri-iodothyronine on the ratios in mitochondria is shown in Table II. Tri-iodothyronine was added to the mitochondria immediately prior to the addition of ADP. The percentage decrease in the ratio (right-hand column) demonstrates the inefficiency of the mitochondria in maintaining the normal ratio as a function of the concentration of triiodothyronine.

With regard to the phenomenon of respiratory control as applied to intact cells, I shall give some data on the glucose-oxygen stoichiometry in ascites tumour cells. The addition of glucose to these cells causes an increase and, after 30–60 seconds, a strong blockage of oxygen uptake as

Table II (HESS)

INFLUENCE OF TRI-IODOTHYRONINE ON ATP/ADP RATIO IN MITOCHONDRIA

Expt. No.	*Tri-iodothyronine* 10^{-6}M	$\Sigma ATP+ADP$	*ratio ATP/ADP*	*Percentage decrease*
1	—	0·4	7·6	—
2	1·0	0·4	7·0	8
3	3·0	0·3	6·4	16
4	5·0	0·4	5·6	26
5	7·0	0·3	1·3	83

shown in 1955 (Chance, B., and Hess, B. (1955). *J. cell. comp. Physiol.*, **46**, 358). Prof. Chance has already mentioned the Q_{O_2} values for this. The underlying mechanism of these effects consists in a release of ADP by the phosphorylation of glucose, then an activation of oxidative phosphorylation through this ADP and, finally, an inhibition of respiration after reconversion of all available ADP to ATP in the mitochondria. Using small amounts of glucose, without exhausting the ATP pool at the hexokinase site, this blockage can be used as a titration point for determination of a glucose/oxygen ratio (see also Chance, B., and Hess, B. (1959). *Science*, in press). Since ADP, through this mechanism, is quantitatively taken up from the acceptor of the oxidative phosphorylation, the value of the ratio should be about 3·0 according to the ADP/O ratio of isolated mitochondria. In Table III, some data (experiments 1–7) are collected which were obtained by measurement of the oxygen uptake by means of the rotating platinum electrode after addition of known amounts of glucose.

Table III (HESS)

DIRECT TITRATION GLUCOSE/OXYGEN RATIO

Expt. No.	*Glucose* µM/*sample*	*Oxygen* µ*atoms/sample*	*Ratio*
1	0·1	0·042	2·4
2	0·2	0·074	2·7
3	0·2	0·071	2·8
4	0·3	0·12	2·5
5	0·3	0·1	3·3
6	0·4	0·15	2·6
7	0·4	0·11	3·5
8	0·3	0·13	2·3*
9	0·4	0·16	2·5*
10	0·5	0·15	3·3*

* In presence of iodoacetate (0·8 mM).

When one considers the increasing amounts of glucose added and the increasing amounts of oxygen taken up, it is obvious that when plotted they form a straight line. In order to define the pathway of this metabolic interaction, the experiments have been repeated in the presence of

0·8 mM iodoacetate as an adequate inhibitor of glyceraldehyde-3-phosphate dehydrogenase. Under these conditions the glucose/oxygen ratio has an average value of 2·7 in agreement with our theory. Table IV

Table IV (HESS)

GLUCOSE/OXYGEN RATIO
(CHEMICAL DETERMINATION OF GLUCOSE)

Expt. No.	*Ratio**
1	3·3
2	2·5
3	1·7
4	2·2
5	2·7
6	2·0
7	2·0
8	3·7
9	1·1†
10	0·9†
11	0·7†

* Ratio: $\frac{\text{moles of glucose}}{\text{atoms of oxygen}}$ per 20 seconds. Initial glucose concentration 10 mM.
† Dicoumarol added, 50 μM.

shows similar data obtained in the presence of excess glucose. In these experiments the initial disappearance of glucose (20 sec.) is measured by chemical determination and correlated with the corresponding oxygen uptake followed with the platinum electrode. The average value of the glucose/oxygen ratio is 2·5. In consideration of the inorganic phosphate taking part in this stoichiometry, the amount of glucose should be equivalent to an amount of phosphate taken up through oxidative phosphorylation. This has been verified and Fig. 1 (p. 126) shows the kinetics of inorganic phosphate after addition of a known amount of glucose. In these experiments, the ascites tumour cells are suspended in a phosphate-free medium so that analysis of the centrifuged cells would show the disappearance of the intracellular phosphate caused by glucose addition. Fig. 1 shows the disappearance of phosphate during glucose utilization for 0·25 and 0·5 μM glucose. A comparison of the scale of the ordinate with that of the initial glucose concentration added shows a glucose/phosphate stoichiometry of approximately 1/1 in confirmation of our theory. It is interesting that the phosphate level is not steady following the utilization of glucose but rises again presumably due to various dephosphorylation processes.

In summary, these experiments demonstrate the possibility of measuring the efficiency of phosphorylation in intact cells.

Aldridge: To return to Dr. Racker's point: if we are to use information from Warburg experiments to get information about how the mitochondria behave in the cell, we have to think rather carefully about the media and conditions under which we examine them. The results which I have already mentioned showing fourfold stimulation were obtained

by using an electrolyte medium of an osmolarity equal to that in the cell (0·3–0·32 osmolar). The ratio of potassium/magnesium/inorganic + nucleotide and phosphocreatine phosphorus was the same as analysis

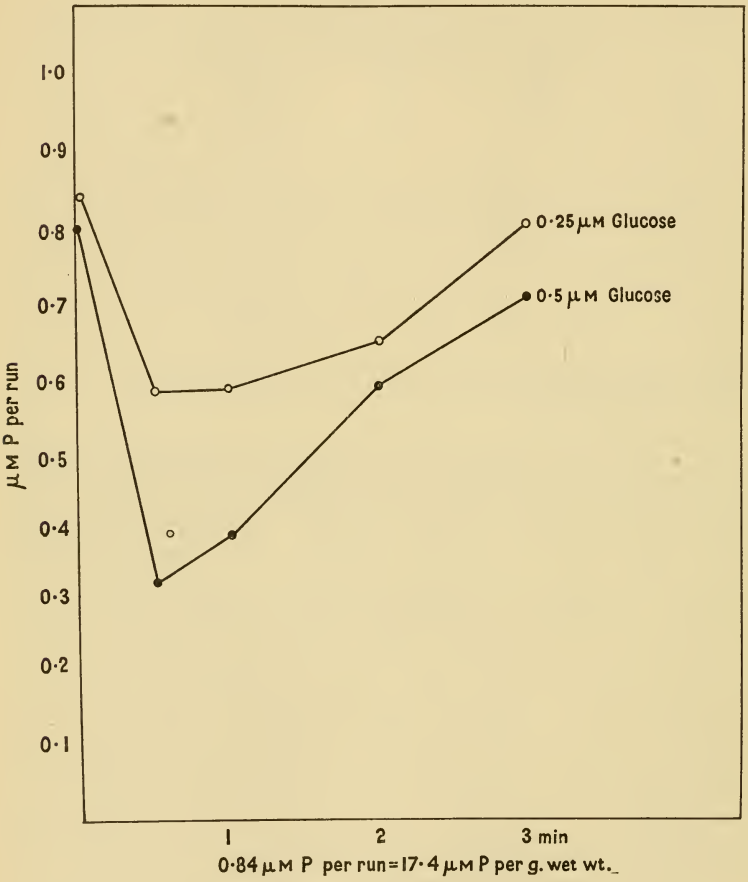

FIG. 1 (Hess). For explanation see text.

of the liver indicated was present in the living cell. The temperature in the experiment was 37°. Although I would not commit myself to saying that the respiratory control in the whole cell only gives a value of four times stimulation, nevertheless it is open to question whether respiratory control is complete in the whole cell. It is agreed that respiratory

control is present in the whole cell, but the extent of it is largely undecided.

Chance: One of the specific objects of our experiments was an attempt to determine directly whether there was a metabolic state which would demonstrate the extent of respiratory control of the living cell. With yeast, it was clear that we were not able to demonstrate anything better than a 50 per cent change of respiration from activity to rest. With the ascites cell we were able to get ratios of 20-fold or more for cells about 30 seconds after their removal from the mouse. I do not think that anyone would question that a muscle will give a ratio of at least 50, perhaps more in insect flight muscle. The thesis that a mitochondrion in a living cell can exhibit a very high ratio of respiratory rates in the presence of phosphate acceptor or phosphate compared to that in the absence of either of these substances, is probably a valid one. One has to consider that perhaps the environment of the mitochondrion within the cell is much more favourable than we can obtain in our polarograph or in the Warburg manometer.

McIlwain: Prof. Chance reported, at the beginning of his talk, the concentrations of reactants to which respiratory processes were sensitive. He pointed out that the processes were sensitive to ADP levels of the order of 20 μM, whereas with inorganic phosphate, sensitivity of the respiratory processes was in a different concentration range of some mM. It might appear from this that the two controlling factors came into operation at different stages of breakdown of ATP. I would like to point out the way in which the presence of the enzyme creatine phosphokinase actually operates to bring these two reactants into action at the same time, because of the nature of the equilibrium at the kinase:

$$\mathrm{CrP} + \mathrm{ADP} \rightleftharpoons \mathrm{Cr} + \mathrm{ATP}$$

The reaction normally goes almost entirely to the right. This gives an explanation of how, in neural or muscular tissues, there can be considerable breakdown of ATP and yet concentrations of ADP remain of the order of 10–20 μM: for the tissue contains creatine phosphate concentrations of perhaps 2–3 mM. This means that one can have a breakdown of creatine phosphate through ATP to inorganic phosphate, involving quantities of the order of 1 m-mole of creatine phosphate per g. of tissue, while the change of ADP concentration is of the order of a few μM, which would still leave the systems operating at the normal cell levels of inorganic phosphate, creatine phosphate and ADP. (Incidentally, the balance in production and utilization of labile phosphate in tissues containing the kinase is reflected largely in their level of creatine phosphate, which is perhaps 100 times that of ADP: a point which has analytical conveniences.)

Gutfreund: Prof. Chance, do you not consider that the half-optimum concentrations of ATP, ADP, phosphate, magnesium and calcium are not independent of one another? If the concentration of available ATP is controlled by the magnesium concentration, then it is possible to get half-optimum Mg^{2+} concentration at a fixed concentration of all

phosphate compounds. The same applies to calcium. If calcium is added, it will compete with magnesium and will form an ATP complex, which is not available, and there will be a loss of respiratory control due to the addition of calcium. Also, one can then get the interesting effect which Prof. Chance obtained, i.e. by adding small amounts of calcium, respiratory control is only temporarily lost, and by adding more calcium one gets complete loss of respiratory control. This could be accounted for if some of the ATP is made unavailable or more slowly available, due to a shift of equilibrium by the addition of calcium; and if more calcium is added, the equilibrium of available ATP is upset so that there is a complete loss of respiratory control.

Holzer: Prof. Chance explained his experiment with yeast and glucose by the assumption that the ATP concentration in starved yeast cells is very low, and that the ADP concentration is high. We have measured ADP and ATP concentrations in yeast cells (Holzer, H., Witt, J., and Freytag-Hilf, R. (1958). *Biochem. Z.*, **329**, 467). Most surprisingly, we found that the concentration of ATP in aerobic starved yeast is as high as in glucose-oxidizing yeast cells. If glucose is added under aerobic conditions to starved yeast, the following is observed (Holzer, H., and Freytag-Hilf, R. (1959). In preparation): during the first 7 seconds ATP decreases very rapidly, increases again, and then a steady state concentration is achieved, which is approximately as high as the steady state concentration prior to glucose addition. The time during which these changes in concentration occur is about 10–20 seconds, depending on the temperature (we measured at 25°). The ADP changes are the exact reversal of those of ATP. From this it results that in starved yeast the total concentration of ATP (without respect to compartmentation) is as high as in the steady state of glucose-oxidizing yeast.

Chance: We do agree in certain respects, but it is important to point out that Prof. Holzer and I measure nucleotide concentrations at different places. Also, we pretreat the yeast with a low ethanol concentration to obtain a minimal ADP concentration and then add glucose. The ADP that we measure is that ADP accessible to the mitochondria, and its rise in concentration upon glucose addition stimulates respiration. The nucleotide that Prof. Holzer measures is the sum of the cytoplasmic and mitochondrial concentration i.e. not only is there generation and utilization of ATP on addition of glucose but there is also a movement of ATP from the cytoplasm to the mitochondria.

Slater: Prof. Chance, did you measure the K_m for ADP in the presence of inorganic phosphate, and was the K_m for inorganic phosphate measured in the presence of ADP?

Chance: Yes; we gave the K_m for ADP in presence of substrate and phosphate, so that it was the dynamic K_m referring to maximal respiratory activity. The K_m for phosphate was determined in the presence of ADP and substrate.

Lipmann: Did you wait long enough to obtain a steady state? The ^{32}P equilibration in mitochondria takes a long time.

Chance: Ours were steady state values, i.e. we waited long enough until either maximal respiration or maximal change of the respiratory

carrier had been established. Prof. Lipmann's point was very well taken with regard to phosphate: it does take a while to achieve a steady state with phosphate. Perhaps it is almost two minutes before we get the steady state.

Slater: The K_m values which I quoted in answer to Dr. Hess' question (p. 85) are the same K_m's measured under exactly the same conditions. The figures are very similar; I mentioned a K_m of 70 μM, corresponding to your 56 μM.

Siekevitz: ADP must be added to mitochondria in order to get a stimulation of respiration. Yet mitochondria always have ADP there when we extract them with acid and measure the amount of ADP. ADP, ATP and AMP are present, according to our information. We think that the ADP is bound in the mitochondria and unavailable for stimulating oxidation. We have never found, during oxidative phosphorylation, any increase or decrease in ADP or ATP within the mitochondria. They always seem to be at the same levels. It has been found that they will take up inorganic phosphate; i.e. the phosphate concentration within the mitochondria can be increased several fold (Bartley, W., and Davies, R. E. (1954). *Biochem. J.*, **57**, 37; Siekevitz, P., and Potter, V. R. (1955). *J. biol. Chem.*, **215**, 237). This finding might have something to do with the difference in the K_m values that you get, and also with the lag period obtained during the equilibration when you add phosphate, as against the addition of ADP when no lag period was observed. ADP reacts immediately, because even if there is ADP in the mitochondria, it is bound and unavailable; it might be there at just below threshold level. If you add phosphate, you may have to increase the phosphate concentration in the mitochondria to a certain level before respiration is stimulated.

Chance: You have put your finger on the central question of chemical analysis of mitochondria and intact cells. Most analyses simply do not show the concentrations of ATP/ADP which our K_m values suggest, except for the data that Dr. Hess has presented (see Table I, p. 123).

Hess: The criterion for this type of test is the state of mitochondrial respiration. The mitochondria should scarcely respire in the presence of substrate and endogenous ADP or added ADP after reaching the steady state equilibrium of the resting condition which we call state 4 (ADP being the rate-limiting factor). The respiration rate must be very small in this state; in other words, the mitochondria must have respiratory control. The data I have discussed (see Tables I and II, above) are obtained through analysis of mitochondria in this state, which was detected by means of the oxygen electrode. We have done this analysis many times, and can also titrate the ATP down by adding tri-iodothyronine (see Table II, above). I wonder whether Prof. Siekevitz has kept his mitochondria in this state of respiratory control. Then the discrepancy between his and our data would be understandable.

CONTROL POINTS IN PHOSPHORYLATING RESPIRATION AND THE ACTION OF A MITOCHONDRIAL RESPIRATION-RELEASING FACTOR*

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THIS paper outlines recent work in this laboratory on the sequence and character of the terminal reactions by which adenosine triphosphate (ATP) is formed during respiratory chain phosphorylation, the site of action of some classical uncoupling agents, and the effects of a naturally occurring mitochondrial agent capable of controlling some aspects of the coupling of phosphorylation to respiration.

Exchange reactions of ATP and the sequence of coupling reactions

Earlier work has shown that submitochondrial fragments prepared with digitonin contain more or less intact respiratory chains and couple phosphorylation of adenosine diphosphate (ADP) to electron transport at three sites with reasonably high efficiency (Cooper and Lehninger, 1956*a* and *b*; Devlin and Lehninger, 1956, 1958; Devlin, 1959; Lehninger *et al.*, 1958). Such preparations catalyse two exchange reactions of ATP in the absence of net electron transport which are reflections of the energy-coupling mechanism.

In the ATP-$^{32}P_i$ (P_i = inorganic phosphate) exchange reaction, orthophosphate labelled with ^{32}P is incorporated into the terminal phosphate of ATP (Cooper and Lehninger, 1957*b*), and this reaction may proceed to isotopic equilibrium.

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The reaction is inhibited by 2 : 4-dinitrophenol (DNP) and other uncoupling agents with about the same sensitivity as oxidative phosphorylation. The rate of this exchange reaction is maximum when the respiratory carriers of the preparation are maintained in the oxidized state (Wadkins and Lehninger, 1957). ADP has been demonstrated to be an essential component of the ATP-$^{32}P_i$ exchange reaction (Cooper and Lehninger, 1957*b*).

A second exchange reaction of ATP is catalysed by the phosphorylating respiratory fragments, namely the ATP-ADP exchange, in which ^{32}P- or ^{14}C-labelled ADP is incorporated bodily into ATP in a reversible manner in the absence of net electron transport (Lehninger *et al.*, 1958; Cooper and Lehninger, 1957*b*; Wadkins and Lehninger, 1958). The ATP-ADP exchange is very rapid (faster than the ATP-$^{32}P_i$ exchange) and proceeds to isotopic equilibrium. The ATP-ADP exchange is also inhibited by DNP in freshly prepared phosphorylating fragments, showing its relationship to oxidative phosphorylation. The DNP-insensitive ATP-ADP exchange reactions associated with other phosphate-transferring reactions, as in glutathione and glutamic synthetases, the succinate-linked P-enzyme, etc. are absent from the fragment preparations. The ATP-ADP exchange of oxidative phosphorylation requires Mg^{2+} but no other dialysable component.

The important observation was made that inorganic phosphate is not required for the ATP-ADP exchange nor does it affect the rate of the exchange over a 5000-fold range of concentration (Lehninger, *et al.*, 1958; Wadkins and Lehninger, 1958), suggesting that inorganic phosphate is taken up in another reaction, probably preceding the uptake of ADP. This suggestion was given very firm support by the finding that ageing of the enzyme fragments at 2° for 48 hours or longer caused complete loss of the ability to catalyse the incorporation of $^{32}P_i$ into ATP, but resulted in no loss of activity of the ATP-ADP exchange. Thus, inorganic phosphate must be incorporated by a rather labile reaction which precedes the entry of ADP.

These findings on the ATP exchanges may be considered in the context of a mechanism of energy coupling in oxidative phosphorylation which we postulated earlier, as follows (Lehninger, *et al.*, 1958; Cooper and Lehninger, 1957*b*; Lehninger, 1955):

(1) $\text{CARRIER}_{\text{RED.}} + \text{X} + \text{carrier}'_{\text{ox.}} \rightleftarrows \text{CARRIER}_{\text{OX.}} \sim \text{X} + \text{carrier}'_{\text{red.}}$

(2) $\text{CARRIER}_{\text{OX.}} \sim \text{X} + \text{P}_{\text{i}} \rightleftarrows \text{CARRIER}_{\text{OX.}} + \text{P} \sim \text{X}$

(3) $\text{P} \sim \text{X} + \text{ADP} \rightleftarrows \text{ATP} + \text{X}$

In this formulation, X is postulated to be an enzyme protein interacting with a specific carrier (i.e. $\text{CARRIER}_{\text{RED.}}$) during its oxidation to produce a "high-energy" compound with the oxidized form of the carrier ($\text{CARRIER}_{\text{OX.}} \sim \text{X}$). This "charged" carrier is now postulated to undergo a phosphorolytic reaction, presumably by nucleophilic attack of phosphate at the "high energy" bond to form $\text{P} \sim \text{X}$ (a phospho-enzyme). The $\text{P} \sim \text{X}$ so generated donates its P to ADP. In principle the reaction pattern was first postulated by Lipmann (1946) and has since been elaborated by many others. As written, it is a skeleton mechanism; it is not excluded that intermediate group-transfer reactions may occur between reactions (1) and (2) and between (2) and (3), such as those postulated by Chance and Williams (1956). The formulation of reactions (1), (2) and (3) involves the oxidized form of the carrier as the high-energy or charged form, for which we give evidence elsewhere (Wadkins and Lehninger, 1957, 1958). On the other hand, Chance has favoured a thermodynamically equivalent formulation which utilizes the reduced form as the high-energy form (Chance and Williams, 1956; Chance and Hollunger, 1957).

This reaction scheme readily accounts for the findings on the ATP exchanges. It is seen that this formulation provides for incorporation of $^{32}\text{P}_{\text{i}}$ into ATP in the absence of *net* electron transport at maximum rates when the carriers are oxidized. This is made possible by reversible phosphorolysis of the high-

energy linkage in the pre-existing $CARRIER_{OX.} \sim X$. The scheme also shows that ADP is a necessary reaction partner for incorporation of $^{32}P_i$. On the other hand, it accounts for the finding that reversible incorporation of ADP may proceed in the absence of phosphate and that the incorporation of $^{32}P_i$ may be inactivated by ageing [elimination of reaction (2)] leaving reaction (3) intact. It is concluded that the terminal reaction of oxidative phosphorylation has the form of reaction (3), where $P \sim X$ is a bifunctional phosphate-transferring enzyme. The findings exclude an alternative formulation involving an $ADP \sim X$ complex (Wadkins and Lehninger, 1959).

The experiments on the exchange reactions of ATP thus establish the sequence of entry of orthophosphate and ADP in the mechanism of energy coupling shown in reactions (1), (2) and (3). These findings are given additional support by the following experiments.

Site of action of uncoupling agents

Measurement of the effects of a variety of different uncoupling agents on the rate of the two ATP exchanges, the P/O ratio during oxidation of β-hydroxybutyrate, as well as the ATPase activity (Cooper and Lehninger, 1957*a*) [a reflection of the reverse of reactions (7) and (6), followed presumably by hydrolysis of carrier $\sim$ X or its energetic equivalent] yielded the data summarized in Table I. Freshly prepared digitonin subfragments were studied.

The three agents DNP, dicoumarol and gramicidin uncouple oxidative phosphorylation, inhibit both exchange reactions of ATP and stimulate ATPase activity, demonstrating that they act on a component common to all the partial reactions of oxidative phosphorylation.

Thyroxine and its analogues, and also Ca^{2+}, although potent uncoupling agents in intact mitochondria, do not uncouple oxidative phosphorylation in the digitonin fragments, nor do they affect any of the partial reactions at concentrations which uncouple in intact mitochondria. They have a mode of

Table I

EFFECT OF UNCOUPLING AGENTS ON PARTIAL REACTIONS OF OXIDATIVE PHOSPHORYLATION IN FRESH DIGITONIN FRAGMENTS

Uncoupling agent	*Concentration* M	*Uncoupling activity*	*Inhibition of ATP-$^{32}P_i$ exchange*	*Inhibition of ATP-ADP exchange*	*Stimulation of ATPase*
DNP	5×10^{-5}	+	+	+	+
Dicoumarol	1×10^{-5}	+	+	+	+
Gramicidin	1×10^{-5}	+	+	+	+
Sodium azide	1×10^{-3}	+	+	0	Inhibition
Ageing at 2°	—	+	+	0	Inhibition
Ionic strength (KCl)	0·4	+	+	0	Inhibition
Sucrose	0·5	+	+	0	Inhibition
Arsenate	1×10^{-2}	+	+	0	0
Methylene blue	1×10^{-5}	+	+	0	+
p-Chloromercuribenzoate	1×10^{-3}	+	+	+	Inhibition
L-Thyroxine	1×10^{-5}	0	0	0	0
Ca^{2+}	1×10^{-3}	0	0	0	0

action in intact mitochondria which is obviously different from that of DNP, dicoumarol, and gramicidin; elsewhere we have shown that thyroxine causes changes in the mitochondrial membrane and consequent swelling, possibly by an interaction with a bound form of diphosphopyridine nucleotide (DPN) (Tapley, Cooper and Lehninger, 1955; Lehninger, 1956; Lehninger, Ray and Schneider, 1959).

There are three agents which uncouple phosphorylation and inhibit the ATP-$^{32}P_i$ exchange, but which do not inhibit the ATP-ADP exchange, namely azide, arsenate and methylene blue. Clearly, the action of these three uncoupling agents differs from that of DNP, dicoumarol and gramicidin; their action must be in either reaction (1) or reaction (2) (or an intervening reaction) but not in reaction (3) itself. The action of arsenate is already known to be that of a competitor of phosphate; presumably its site of action is in reaction (2). Methylene blue by its chemical nature can be postulated to replace or alter reactivity of a carrier component in reactions (1) or (2). *p*-Chloromercuribenzoate (PCMB) differs from all the rest of the agents since it inhibits all the partial reactions, including ATPase activity.

A second type of experiment made it possible to pinpoint more closely the site of action of different uncoupling agents in the coupling mechanism. Ageing of the submitochondrial fragments at 2° for 72 hours has been found to cause complete loss of coupling activity, complete loss of ATP-$^{32}P_i$ exchange activity, complete loss of ATPase, but relatively insignificant loss in the activity of the ATP-ADP exchange, indicating that reaction (7) can proceed independently of the other partial reactions of coupling (Wadkins and Lehninger, 1959).

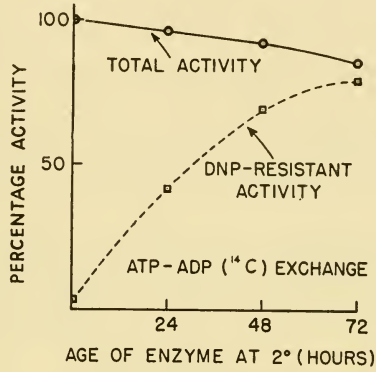

Fig. 1. Loss of sensitivity of the ATP–ADP exchange to DNP on ageing of phosphorylating fragments.

However, the very important finding was made that such ageing of the particles, although causing no significant loss of ATP-ADP exchange activity, does cause loss of the sensitivity of this reaction to inhibition by DNP and dicoumarol, the rate of loss of the sensitivity being exactly parallel to the rate of inactivation of the ATP-$^{32}P_i$ exchange (Fig. 1) (Wadkins and Lehninger, 1959). This finding clearly indicates that the ATP-ADP exchange *per se* is inherently insensitive to inhibition by DNP and that DNP-sensitivity is conferred on it by a factor which is labile to ageing. Since the ATP-ADP exchange has a reaction component (P $\sim$ X) which is shared with the preceding reaction (2) in the hypothesis, it appears probable

that DNP causes some limitation on, or removal of, $P \sim X$ by an action on either reaction (1) or reaction (2) in fresh preparations. Ageing thus causes inactivation of a reaction located between the site of action of DNP and the ATP-ADP exchange. It is clear also that the effect of ageing is not the equivalent of the action of DNP, as has been suggested by others, since ageing causes loss of ATPase activity of the mitochondrial fragments whereas DNP stimulates it.

In similar experiments it has been found that sensitivity of the ATP-ADP exchange to dicoumarol is similarly abolished by ageing, suggesting that it has a site of action near that of DNP, but not necessarily at the same point.

A third type of experiment has confirmed these implications and made it possible to establish the approximate sites of action of azide and of high concentrations of neutral salts and sucrose, which are agencies causing uncoupling and inhibition of the ATP-$^{32}P_i$ exchange and ATPase. Data in Table I show azide to be an uncoupling agent and an inhibitor of the ATP-$^{32}P_i$ exchange but that it inhibits ATPase activity, and has no effect on the ATP-ADP exchange, indicating that it has a site of action different from that of DNP. This was not only confirmed, but the approximate site of action of azide was defined by the experiments summarized in Table II. It is seen that azide, although not inhibitory to the ATP-ADP exchange, abolishes the sensitivity of the ATP-ADP exchange to DNP and also to dicoumarol in fresh submitochondrial fragments. Therefore azide has a site of action which lies between the site of action of DNP and the terminal ATP-ADP exchange. Similarly, azide was found to abolish the stimulating effect of DNP on ATPase activity, an observation wholly consistent with this conclusion (Wadkins and Lehninger, 1959). It is possible that azide inhibits a phase of reaction (2) which is also easily inactivated by ageing.

Similar experiments have shown that DNP-sensitivity of the ATP-ADP exchange, as well as DNP-stimulation of ATP-ase activity, is prevented by 0·3 M KCl and by 0·5 M sucrose. Clearly, these agents must also act at or near the site of action

Table II

ACTION OF AZIDE ON SENSITIVITY OF ATP-ADP EXCHANGE TO DNP AND DICOUMAROL

System contained 0·003 M-ATP, 0·002 M-ADP-^{14}C, digitonin fragments (50 μg. N), pH 6·8, in total volume of 0·50 ml. Incubated 20 minutes at 23°.

Additions	*ATP-ADP exchange mμmoles ATP-^{14}C formed*
(1) None	158
5×10^{-4} M DNP	16
1×10^{-3} M NaN_3	154
5×10^{-4} M DNP + 1×10^{-3} M NaN_3	149
(2) None	178
1×10^{-4} M dicoumarol	12
1×10^{-3} M NaN_3	174
1×10^{-4} M dicoumarol + 1×10^{-3} M NaN_3	169

of azide or of ageing. PCMB must act on reaction (3), but it is not excluded that it may also react at other points in the scheme.

The relationships developed are summarized schematically in Fig. 2. It must be repeated that additional group-transfer reactions may intervene between reactions (1) and (2) and

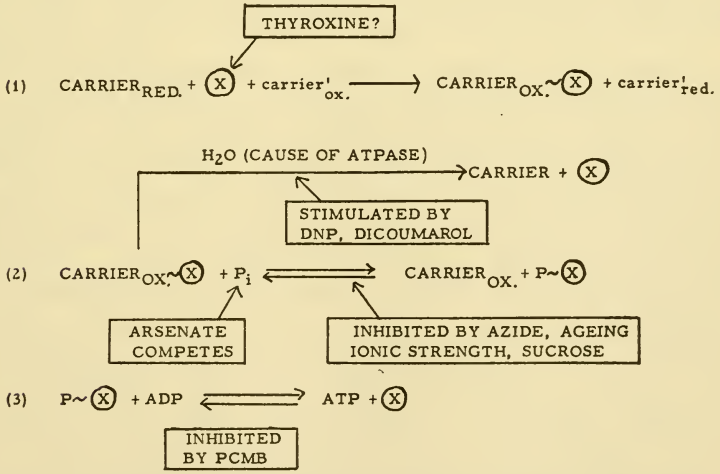

FIG. 2. Relative sites of action of uncoupling agents in postulated mechanism.

between (2) and (3). However, if so, the *relative* sites of action of the uncoupling agencies must be in the sequence shown, regardless of the number of intervening reactions.

We have recently found it possible to dissociate the enzyme catalysing reaction (3) in soluble form from the digitonin fragments and have purified it substantially by classical fractionation methods. It is quite stable and requires no dialysable cofactor other than Mg^{2+}. It is thus the first enzyme of the coupling mechanism reported in soluble and purified condition and will provide access to the preceding reactions of energy coupling to the respiratory chain through *in vitro* reconstruction methods utilizing purified enzyme components. The soluble enzyme is inhibited by PCMB, but not by DNP, dicoumarol, gramicidin, azide, KCl or sucrose. These observations not only confirm the findings on the digitonin fragments, but also provide an unequivocal landmark for identification of sites of action of uncoupling agents.

Naturally occurring controlling factors in mitochondria

There have been frequent suggestions that agents exist in the cell which act as physiological uncoupling agents, which can lower the efficiency of energy coupling and also stimulate respiration by releasing it from its rate-dependence on ADP and phosphate. Thyroxine has been visualized as one such agent and our more recent findings indicate that it causes a "dislocation" of a respiratory carrier, a bound form of DPN (Lehninger, Ray and Schneider, 1959). However, it appears probable that mitochondria themselves must also contain uncoupling and/or respiration-releasing factors, since in fresh mitochondria ADP and phosphate are obligatory for respiration to occur whereas in aged mitochondria neither ADP nor P_i is required for maximum rates of respiration, nor do they significantly affect the rate. Mechanical or chemical disruption of mitochondria also often produces similar transformations from phosphorylating ADP-controlled electron transport to non-phosphorylating respiration independent of ADP. Such findings pose rather fundamental

questions (Lehninger, 1955). One explanation is that mitochondria contain two sets of respiratory carriers, one set of which is obligatorily coupled to uptake of ADP and P_i, and the other purely non-phosphorylating; in fresh mitochondria the former set dominates, in disrupted mitochondria the second set replaces the first. This appears to be an unlikely mechanism. A second alternative is that the three specific carriers which normally participate in energy coupling and thus in respiratory control by ADP are "shunted out" of the respiratory chain following mitochondrial damage, with formation of non-phosphorylating "short-circuits" around the normally phosphorylating carriers (as may be true in the case of cytochrome *b*). These shunts could be made possible by the relative non-specificity of electron donors and acceptors in the chain. A third possibility is that the same set of carriers participates in both non-phosphorylating and in phosphorylating respiration, but that activation of an uncoupling enzyme in disrupted mitochondria releases the chain from dependence on ADP and P_i. There is some evidence supporting both the second and third alternatives and it is possible that both mechanisms are involved in the general phenomenon.

Mitochondrial factors which uncouple phosphorylation and stimulate ATPase have been described by Pullman and Racker (1956) and by Polis and Shmukler (1957), whose factors may well be identical. The latter authors have presented evidence that their factor, called mitochrome, is a haemprotein. More recently Hülsmann, Elliott and Rudney (1958) have shown that the uncoupling activity of such mitochrome preparations resides in an *iso*octane-extractable fraction of the protein. Such *iso*octane extracts of mitochrome have been reported to uncouple phosphorylation in intact rat liver mitochondria, to stimulate ATPase activity and to inhibit the ATP-$^{32}P_i$ exchange reaction, properties which are in general similar to those of DNP. The activity of these factors is characteristically abolished by serum albumin, which is also capable of restoring phosphorylation in aged

preparations of mitochondria (Pullman and Racker, 1956; Polis and Shmukler, 1957; Stern and Timonen, 1955; Beyer and Glomset, 1956) and in fly mitochondria (Sacktor, 1954).

We have recently separated a mitochondrial factor having some properties in common with mitochrome and the *iso*-octane-extractable factor of Hülsmann and co-workers, but which has other rather striking effects on electron transport and coupled phosphorylation which have not yet been described and which may be important in intramitochondrial control of respiration (Remmert and Lehninger, 1959). The factor to be described may contain a more native form of mitochrome or its *iso*octane-soluble activity described above; but it is probable that it is a mixture of factors affecting energy coupling.

Freshly prepared rat liver mitochondria are subjected to 9 kcyc. sonic oscillation and the débris is extracted with dilute sucrose-KCl solutions. The extract is clarified by centrifugation at 115,000 ***g*** for 30 minutes and then dialysed exhaustively. Protein precipitating from this extract when the pH is adjusted to 5·5 represents the basic starting material for our tests.

When tested in relatively high concentrations, this soluble mitochondrial protein fraction (called R factor) causes uncoupling of phosphorylation from oxidation of β-hydroxybutyrate, inhibition of the ATP-$^{32}P_i$ exchange reaction, and stimulation of ATPase activity, as is shown by the data in Table III. The preparation of R factor itself shows no activity in oxidative phosphorylation or in the ATP-$^{32}P_i$ exchange and only negligible ATPase activity. These properties of the R factor preparation, taken at face value, suggest it is a naturally occurring uncoupling agent present in fresh mitochondria (but presumably in "masked" form) which is released on sonication. In the reactions shown in Table I it behaves like DNP and gives the same effects as preparations of mitochrome or its *iso*octane-soluble component. However, further study shows it to be very different from DNP and possibly different from the materials described by others.

It is now well known that in the absence of ADP freshly prepared mitochondria respire very slowly, but that addition of DNP causes release of respiration. Such release of respiration presumably is caused by the discharge of high-energy intermediates of the carriers by DNP, such as the carrier $\sim$ X of reaction (1). These phenomena are also observed with the digitonin fragments of mitochondria; DNP causes stimulation of ADP-less respiration to a limiting level which is exactly the maximum rate given by addition of ADP itself. Preparations

Table III

Action of R factor on partial reactions of oxidative phosphorylation

Test systems for the assays as described by Wadkins and Lehninger (1959). The concentration of R factor preparation added was approximately three times the concentration just necessary to produce maximal respiratory stimulation (see text).

Reaction	*Quantity measured*	*Control*	*Addition of R factor*
(1) Respiration in presence of ADP	Δmμmoles acetoacetate	852	870
(2) Coupled phosphorylation	Δmμmoles $AT^{32}P$	1262	493
(3) P/O	moles $AT^{32}P$ per mole acetoacetate	1·48	0·57
(4) ATP-$^{32}P_i$ exchange	Δmμmoles $AT^{32}P$	256	65
(5) ATPase	Δmμmoles P_i	1069	1670

of the R factor also show this action, as shown in Table IV, the release of respiration being roughly proportional to the amount of R factor added.

However the respiration-releasing activities of DNP and R factor are quite different in nature. A concentration of DNP just adequate to release respiration maximally, when added to a parallel test system containing ADP, produces substantially complete uncoupling of phosphorylation. On the other hand, concentrations of R factor preparation which just produce complete release of respiration in an ADP-less system produce only partial and often very little uncoupling of phosphorylation when tested in a system containing ADP, as is shown by the P/O ratios in Table IV. It is of significance that different preparations of R factor vary in their relative

Table IV

RESPIRATION-RELEASING ACTION OF R FACTOR

Test system contains digitonin fragments (100 μg. N), 0·01 M β-hydroxybutyrate, 0·01 M phosphate labelled with ^{32}P, pH 6·5, water, and additions shown to volume of 3·0 ml. Incubated 30 minutes at 23°. ADP was added at 0·0024 M as shown.

	Rate of respiration mμmoles acetoacetate		
Additions	*−ADP*	*+ADP*	*P : 2e**
None	108	435	1·8
0·1 ml. R factor	240	440	1·8
0·2 ml. R factor	360	428	1·7
0·4 ml. R factor	**442**	**435**	**1·6**
0·8 ml. R factor	452	448	0·9
1·2 ml. R factor	462	460	0·3
None	79	347	1·9
1×10^{-6} M DNP	189	340	0·9
1×10^{-5} M DNP	**330**	**320**	**0·1**
1×10^{-4} M DNP	320	324	0·0

* P : 2e = moles $AT^{32}P$ formed per mole of acetoacetate.

uncoupling and releasing activities. Some preparations release respiration fully with negligible uncoupling, whereas others uncouple substantially but not completely, a behaviour which suggests occurrence of two factors in the preparation. The R factor activity thus differs from that of DNP and all known uncoupling agents we have studied, and the R factor is thus more properly described as a "respiration-releasing" factor. The extra respiration of the digitonin fragments which is stimulated by the R factor is completely inhibited by antimycin A and is also blocked completely by presence of serum albumin. Tests of the site of action in the respiratory chain indicate that the R factor stimulates electron transfer in the span β-hydroxybutyrate to cytochrome *c* and also in the span (ascorbate) cytochrome *c* to oxygen, indicating that its action may be on all three uncoupling sites.

There are as yet insufficient data at hand to define the mode of action of the R factor precisely in terms of the reaction mechanism postulated in Fig. 2. However, it appears necessary to postulate that the factor acts at a point other than

DNP and that its action is prevented by the presence of ADP, possibly in a competitive manner. It may, in fact, be necessary to modify the scheme by adding an additional reaction or reactions. It also appears certain that the crude factor may represent a mixture of factors, which may titrate each other out under certain circumstances. Actually, *iso*octane extraction removes some but not all activity from the preparation, suggesting that it contains the factor described by Hülsmann, Elliott and Rudney (1958), among others. The separation and identification of these factors is in progress.

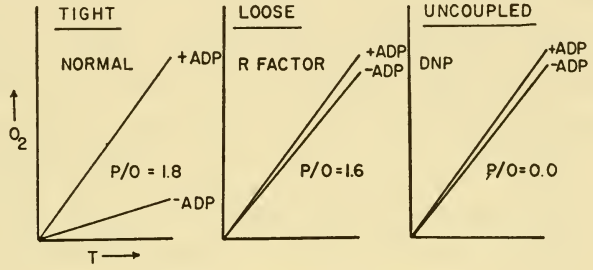

Fig. 3. Types of respiratory coupling and action of R factor and DNP.

It appears quite clear that R factor activity may be a major element in a rather elusive problem, namely the mechanism of "tight" versus "loose" coupling in phosphorylating mitochondrial systems. Figure 3 shows opposite poles of respiratory control produced by ADP and R factor activity. The normal tightly coupled system is shown at the left, with high P/O ratio and a large ADP stimulation of respiration. Addition of R factor produces a "loosely coupled" system in which respiration may proceed at the same rates in the absence or presence of ADP and with nearly normal phosphorylation. The "loosely coupled" respiration shown may very likely be a physiological event. It will be recalled that Hoch and Lipmann (1954) have shown that mitochondria isolated from livers of hyperthyroid rats have normal P/O ratios but

respire at high rates in the complete absence of a phosphate acceptor system, whereas normal mitochondria require the acceptor system for maximum rates of respiration.

Summary

Studies of the properties and requirements of the ATP-$^{32}P_i$ and ATP-ADP exchanges occurring in phosphorylating mitochondrial fragments indicate that phosphate is incorporated in a labile reaction step which precedes the reaction in which ADP is taken up. This conclusion is strongly supported by the finding that $^{32}P_i$ incorporation may be inhibited by azide, ageing, ionic strength and sucrose, without affecting incorporation of ADP. Similarly these agents also abolish the DNP-sensitivity of the ATP-ADP exchange. Location of the relative sites of action of a wide variety of uncoupling agents is thus made possible by such approaches, for which the behaviour of the ATP-ADP exchange enzyme in soluble purified form toward uncoupling agents provides an unequivocal basis.

A soluble protein fraction (R factor) obtained from rat liver mitochondria which may contain mitochrome but which is probably a mixture, uncouples phosphorylation and inhibits the ATP-$^{32}P_i$ exchange at high concentrations. Such R factor preparations release respiration in the absence of ADP. Unlike DNP, however, the R factor releases respiration at concentrations which cause little uncoupling of phosphorylation. Addition of R factor to respiratory fragments thus produces "loose coupled" systems characterized by high P : 2e ratios but no respiratory stimulation by ADP.

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DISCUSSION

Lipmann: I first used the term loose coupling in relation to thyroxine. In our early experiments with Niemeyer and Kennedy, we found this strange absence of the acceptor effect in mitochondria taken out from thyroxinized animals. Then we stated that because in these preparations we still could get P/O ratios which were normal, we like to consider this, together with an absent acceptor effect, as a loose coupling (Hoch and Lipmann, 1954, *loc. cit.*; Lipmann, F. (1956). *In* Enzymes: Units of Biological Structure and Function, p. 444, Ed. Gaebler, O. H. New York: Academic Press). I am very pleased that you have given it a fitting explanation now. I called it loose coupling without really knowing what I was talking about, but you have made some sense of it.

Lehninger: Your experiments were a guide to us; it was evident that there had to be some kind of factor that caused this phenomenon. Very few people have investigated this with uncoupling agents. It could be concerned in the hyperthyroid condition.

Racker: Could this phenomenon be due to a very delicate balance between phosphorylation and ATPase activity or is this possibility out of the question?

Lehninger: We cannot exclude an action of an ATPase but it seems extremely unlikely. The effect cannot be simulated by increasing ATPase activity. Furthermore, it would be necessary then to postulate that the presence of ADP can inhibit ATPase almost completely.

Racker: Pullman, M. E., and Penefsky, Z. J. in our laboratory (1958, *Arch. Biochem.*, 76, 227), have succeeded in separating two distinct fractions from beef heart mitochondria after breakage with glass beads in a Nossal shaker. These fractions appear to be different from those described by Prof. Lehninger. There is one fraction which is particulate and catalyses respiration but does not couple it to phosphorylation. The second factor is soluble and has been purified by ammonium sulphate and protamine fractionation. This factor when added to the particulate fraction allows phosphorylation to be coupled to oxidation. Oxidative phosphorylation in this system is sensitive to dinitrophenol. Curiously enough, the soluble fraction also contains a dinitrophenol-sensitive ATPase activity. Whether this activity is in any way associated with the phosphorylation activity we cannot tell at the present time, but at first sight this soluble factor appears to be different from Prof. Lehninger's fractions, and I am at a loss to fit his and our findings into a common scheme.

Lehninger: It is possible that this fraction represents the whole coupling machinery, including X.

Racker: You mention that the complete system is very unstable and difficult to purify. The soluble fraction is quite stable, particularly in ammonium sulphate.

Lehninger: I would have expected that the second step where ^{32}P enters would be unstable.

Racker: The soluble factor, after several steps of purification, does not catalyse the $^{32}P_i$ exchange and preliminary experiments indicate that it has little if any ADP exchange.

Lehninger: That is very puzzling.

Siekevitz: With regard to the R factor, is there any bound ADP there; could any bound ADP have been released from the particles?

Lehninger: We have not examined that closely. However, the preparations are exhaustively dialysed.

Siekevitz: Where do you visualize the magnesium activation to occur in ATPase? Would it be in the last step or the one before?

Lehninger: The action of magnesium seems to be clearly different. However, magnesium-stimulated ATPase is also inhibited by azide. We feel that reaction 2 is common to both kinds of ATPase; but there must be something different between the action of magnesium and DNP.

Slater: One of the many points I would like to discuss with you is that of the azide inhibition. Following the work of Robertson and Boyer we studied also the effect of azide on the ATPase (Robertson, H. E., and Boyer, P. D. (1955). *J. biol. Chem.*, 214, 295). We found two effects of

azide which could be quite clearly separated from one another (Myers, D. K., and Slater, E. C. (1957). *Biochem. J.*, **67**, 572). Azide acts like a dinitrophenol in that it stimulates the ATPase of freshly prepared liver mitochondria, but also inhibits the DPN-stimulated ATPase or, as you have just said, the magnesium-stimulated ATPase of aged mitochondria. We were able to separate these two effects by a time sequence. The stimulatory effect of azide is a rapid reaction and the inhibitory effect is a slower reaction. You are studying the inhibitory action of azide which is the slow reaction. Have you come across a stimulatory effect?

Lehninger: In intact mitochondria we were fully able to confirm this two-phase effect which Boyer showed, but not with the digitonin fragments—there we got only the one effect (Wadkins and Lehninger, 1959, *loc. cit.*) There does not seem to be biphasic action. I have always been afraid that the ATPase activity of intact mitochondria is much more complex than it is in digitonin fragments. I think there are factors of membrane permeability, compartmentation and so on, which tend to muddle up the issues.

Lipmann: You showed that arsenate competes with phosphate. Does DNP compete with the carrier? We have thought of this because of the competitive inhibition of menadione reductase. Both dicoumarol and DNP inhibit competitively this reductase in liver extracts. We thought that might be the explanation.

Lehninger: I do not think that we have had an appropriate experimental situation to test such a competition.

Racker: It has been found that other flavoproteins, such as the quinone reductase, are inhibited by dinitrophenol (Wosilait, W. D., Nason, A., and Terrell, A. J. (1954). *J. biol. Chem.*, **206**, 271). I wonder whether this may be a rather non-specific effect and not necessarily related to oxidative phosphorylation.

Chance: It is apparent from Prof. Lehninger's mechanism that if azide blocks equation 2 in phosphorylation, then you should have dicoumarol stimulation of respiration above that obtainable by ADP in your scheme (equation 3). I ask this because our kinetic data (Chance and Williams, 1956, *loc. cit.*) show a rate-limiting step in the ADP reaction which should be by-passed with dicoumarol or DNP. Have you done this?

Lehninger: Yes. The work with azide on a respiring system is, of course, difficult because there can be some overlap of those concentrations which uncouple and those which inhibit respiration. But within the limits of this kind of experiment we can do that very nicely. We can put in azide and on top of this get stimulation of respiration by DNP.

Chance: That is above or equal to the level you have obtained with ADP only?

Lehninger: Yes, about equal, we have never been able to get it beyond that.

Chance: Was this with digitonin particles or intact mitochondria?

Lehninger: Digitonin particles.

Chance: I do not believe that this is the case with the azide-treated

liver mitochondria. The speed of the reaction of ADP with the intermediates in phosphorylation is not inhibited by azide concentrations of 400 μM (Chance and Williams, 1956, *loc. cit.*). It is very difficult for me to discuss with you now the relevance of your data on the digitonin particles to our data on intact mitochondria because of this different result. Does this "R factor" act on intact mitochondria?

Lehninger: Yes, it does. Our usual assay is on digitonin particles, but it produces large respiratory stimulation in intact mitochondria.

Chance: Is it any different in effect from lack of magnesium, which I have mentioned already (see p. 101)?

Lehninger: Magnesium additions do not restore respiratory control alone, nor do they affect the action of R factor.

Estabrook: In the light of recent results (MacMurray, W. C., and Lardy, H. A. (1957). *J. Amer. chem. Soc.*, **79**, 6563) on the stimulation of phosphorylation by coenzyme A, I wonder whether your X might be coenzyme A. Have you assayed or analysed the purified enzyme to see if this nucleotide is there?

Lehninger: When the addition of coenzyme A has had no effect, we have not analysed the enzyme because we want to purify it further; the enzyme clearly depends on sulphydryl groups for activity, so we are on the look-out for some connexion with Lardy's findings.

Estabrook: Then your results are different from Lardy's, in that you do not get an effect of coenzyme A.

Lehninger: The digitonin preparation does not respond to coenzyme A, but the sonic particles that Lardy used apparently require coenzyme A.

Holton: The abstract of your paper contains the interesting statement, Prof. Lehninger, that your R factor yields respiration rates which are well beyond those which you can get with ADP or with DNP. This fact cannot be explained by the reaction scheme which you have shown, and necessitates the inclusion of one of the intermediate steps which Prof. Slater had in his scheme. Your result with the R factor corresponds with Dr. Beechey's result with magnesium on the heart sarcosome, which I mentioned earlier, and seems to lead to the same conclusion concerning the mechanism of formation of the DNP-sensitive intermediate in the energy-transfer sequence. Would you like to tell us more about this particular action of the R factor?

Lehninger: Since our abstract was written we found that thorough dialysis removed the substance which gave stimulation beyond the ADP level; its effect could be reproduced by DPN. The more highly purified R factor stimulates exactly to the ADP level.

Hess: Can you get the reverse of reaction 3 from ATP, to the left-hand side of your equation, with the isolated enzyme?

Lehninger: Yes. We can start with labelled ATP and unlabelled ADP e.g. in the presence of azide and digitonin fragments, where there is no ATPase activity, and end up with labelled ADP (Wadkins and Lehninger, 1959, *loc. cit.*

Hess: We do experiments of a similar kind and find that it is very advisable to preincubate the preparation with ATP so that we get,

especially in aged extracts of mitochondria, a far better turnover. It might be that one just fills up this phosphoprotein with ATP. Can you say how much X there is in the system?

Lehninger: We have not tried to determine this, but it must be a very small amount; as a guess I should say of the order of magnitude of the molar concentration of the carriers.

SOME PROBLEMS IN THE CHOICE OF OXIDATIVE PATHWAYS OF CARBOHYDRATE METABOLISM*

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The recognition of the existence of alternative metabolic pathways of the common foodstuffs, particularly of carbohydrate, has posed some rather new and difficult problems for

* Abbreviations used:

ATP, ADP	adenosine triphosphate, adenosine diphosphate
C-1/C-6 ratio	ratio of $^{14}CO_2$ from [1-^{14}C]/[6-^{14}C]glucose
DAB	4-dimethylaminoazobenzene
DHA	dihydroxyacetone
DHAP	dihydroxyacetone phosphate
DPN, TPN	diphosphopyridine nucleotide, triphosphopyridine nucleotide
E–M	Embden-Meyerhof (glycolytic) pathway
F1P, F6P,	fructose-1-phosphate, fructose-6-phosphate,
F1 : $6P_2$	fructose-1 : 6-diphosphate
$\Delta G'$	change of free energy at pH 7 in kg. cal. (Krebs and Kornberg, 1957)
G1P, G6P,	glucose-1-phosphate, glucose-6-phosphate,
G1 : $6P_2$	glucose-1 : 6-diphosphate
D-GA3P	D-glyceraldehyde-3-phosphate
SH7P	sedoheptulose-7-phosphate
Er4P	erythrose-4-phosphate
OAA	oxaloacetate
HMP	hexose monophosphate
ITP, IDP	inosine triphosphate, inosine diphosphate
Pentose P	pentose-5-phosphate
PEP	phospho*enol*pyruvate
6-PG	6-phosphogluconate
RNA	ribonucleic acid
R5P	ribose-5-phosphate
Ru5P	ribulose-5-phosphate
Xu5P	xylulose-5-phosphate
TA	transaldolase
TK	transketolase
UDPG	uridine diphosphate glucose
UTP, UDP	uridine triphosphate, uridine diphosphate

the biochemist. Until comparatively recently most discussions of this kind were concerned with the inter-relationships of the relative amounts of total carbohydrate, fat and protein metabolized, but now in addition it has become necessary to try to analyse the evidence indicating the preponderance of one metabolic pathway over another for one and the same

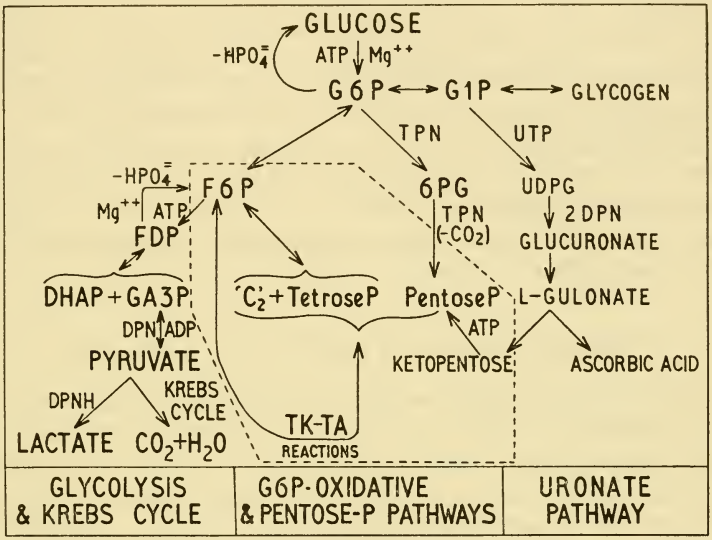


Fig. 1. Pathways of glucose metabolism in animal tissues. Only essential intermediates are included in this diagram.

major metabolite, and to try to discover those factors which determine which of the alternative pathways is that favoured by the cell under varying environmental conditions.

Current views on probable metabolic pathways of carbohydrate metabolism in animal tissues are summarized in Fig.1. For detailed information on the development of knowledge of these routes, a number of recent reviews are available (Racker, 1954, 1957; Gunsalus, Horecker and Wood, 1955; Dickens, 1955, 1958*b*; Horecker and Hiatt, 1958). Microorganisms and plants which present in many cases an even

wider choice of carbohydrate pathways (Gunsalus, Horecker and Wood, 1955), will not be considered in this account. A brief introductory sketch of the main relevant points in each of these pathways will first be given before proceeding to details. As shown in Fig. 1, besides the entry of glucose into the cell, its initial phosphorylation is a primary requirement; the possible pathway via glucose dehydrogenase and further oxidation of the unphosphorylated gluconic acid so formed does not seem to be important in the animal organism (Stetten and Stetten, 1950; Salmony and Whitehead, 1954). The phosphorylation of glucose by the hexokinase reaction requires magnesium ions and consumes 1 equivalent of ATP. The two hexose monophosphate stages which follow thus represent the first key-points for divergence of metabolic pathways, and these stages are therefore "pacemakers" in the nomenclature of Krebs and Kornberg (1957).

The glycolytic and Krebs cycle pathways

The main glycolytic or Embden-Meyerhof (E-M) route is too well known to need comment, except to note that adenosine triphosphate (1 ATP) and magnesium ions are required for the stage of departure from the phosphogluconate pathway at the phosphofructokinase step and that 2 ATP per mole of hexose are regenerated in each of the "substrate-linked" phosphorylations: (*a*) D-GA3P dehydrogenase (which requires 2 moles of inorganic phosphate and in animal tissues is quite specific for DPN), and (*b*) 2 ATP produced from the PEP which results from enolase action on 2-phosphoglycerate. The reduced DPN formed in (*a*) must be reoxidized, either by lactic dehydrogenase plus pyruvate or, aerobically, via the cytochrome system, the latter involving the flavoprotein DPNH-cytochrome *c* reductase and cytochrome oxidase systems. The E-M pathway, besides being historically the first, is also the major pathway of carbohydrate breakdown and the main route leading to energy production in almost all animal tissues. It also provides a route via its reversible

stages for the resynthesis of glycogen from blood lactate or pyruvate, this function being especially important in liver, which according to the well known Cori cycle (Cori, 1931) is the tissue mainly responsible for this resynthesis. The tissue localization of G6Pase, which is active in liver but absent from muscle (Fantl, Rome and Nelson, 1942; Swanson, 1950) prevents breakdown to blood glucose of this intermediate in muscle.

The pyruvate formed in the E-M pathway stands at one of the most important key-points in determining the subsequent route (Figs. 1 and 2). This can be reductive to lactate; a reaction which predominates anaerobically with concomitant reoxidation of the DPNH formed in the glyceraldehyde phosphate oxidative stage. Aerobically, oxidative decarboxylation can yield acetyl coenzyme A (acetyl CoA) which can either undergo condensations and reductions to fatty acids or be oxidized via the Krebs tricarboxylic acid cycle to CO_2 and water; which route is followed depends initially on the amount of acetyl CoA available in relation to the oxaloacetate and to the energy requirements (see Krebs and Kornberg, 1957, for a full discussion). The levels of oxidized DPN^+ and TPN^+ (the latter for the *iso*citric dehydrogenase reaction) and of their respective cytochrome *c* reductases must also be important in favouring a Krebs cycle pathway. The main aerobic source of energy in animal tissues is via this route, being associated primarily with the oxidative decarboxylations of pyruvate, *iso*citrate and α-ketoglutarate and the dehydrogenations of succinate and malate.

Whereas the glycolytic enzymes (like those of the pentose phosphate pathway described below) are located in the soluble fraction of the cell, the Krebs cycle system appears to be localized in the mitochondria, together with the cytochrome electron transport system. Oxidative phosphorylations accompanying the oxidation of DPNH produced in the above reactions can proceed at the maximum rate of 3 moles ATP synthesized per atom of oxygen transported (Lehninger, 1955). The detailed nature of these stages in the oxidative

respiratory chain will be considered by other contributors to this symposium, and here it is merely noted that 15 equivalents of ATP could be formed in the operation of the Krebs cycle proceeding from the level of pyruvate as far as its complete oxidation in one turn of the cycle. The net result is that per molecule of hexose completely oxidized, 32 moles of ATP could be synthesized as compared with only 2 ATP in the conversion of glucose to 2 moles of lactate.

The Krebs cycle, therefore, provides the main energy source of mammalian cells, especially when operating with the glycolytic chain of reactions, since the carbohydrate component of a normal diet generally provides the largest contribution to the total energy source in the intact animal. The individual stages of the Krebs cycle, providing a route for the breakdown (and by producing necessary building materials for the synthesis) of amino acids and fats, are also linked in an essential relationship with the two other main groups of foodstuffs and tissue metabolites. Except for the *iso*citric stage [and even this may be mainly through DPN in mitochondria (Ernster and Navazio, 1957)] the oxidation of carbohydrate by this route is by DPN systems and differs essentially in this respect from the exclusively TPN-linked animal phosphogluconate pathway, to be discussed below.

The glucuronate pathway

Although this recently discovered pathway is not at present considered to have great quantitative importance, it is briefly mentioned here as being the pathway of biosynthesis of ascorbic acid and of the urinary L-xylulose excreted in cases of congenital pentosuria, usually in amounts of from 2–5 g./day (Enklewitz and Lasker, 1933). Both of these metabolites are now believed to be derived from glucuronic acid (or its lactone), the most probable source of which is, as shown in Fig. 1, from G1P reacting with UTP to form UDP-glucose. This uridine nucleotide of G1P can be oxidized at the C-6 of glucose by a two-stage DPN oxidation (Strominger *et al.*,

1957). The UDP-glucuronate so formed is believed to yield free glucuronic acid, which can undergo reduction by a TPNH linked enzyme system acting at the aldehydic C-1 end of the glucose molecule, to give L-gulonic acid. The latter is believed to be oxidized by a DPN system to an intermediate (possibly 3-ketogulonic acid), which can either form ascorbic acid by enolization and lactonization, or L-xylulose by decarboxylation at C-6 of the original glucose molecule (Touster, Hutcheson and Rice, 1955; Bublitz, Grollman and Lehninger, 1957; Ashwell, 1957; Burns and Kanfer, 1957). It is thought that only the latter reaction occurs in species such as man and guinea pig which are unable to synthesize ascorbic acid.

In cases of essential pentosuria, L-xylulose is excreted; otherwise it is reduced by a TPNH-coupled enzyme to xylitol, which then becomes oxidized by a DPN enzyme system of liver to D-xylulose (Hollmann and Touster, 1957). The latter can then be phosphorylated by ATP acting with a specific phosphokinase to give D-Xu5P (Hickman and Ashwell, 1957) which can enter the pentose phosphate cycle, described below, and thus be converted to hexose. These reactions explain why xylitol and D-ribose can both be efficiently incorporated as glucose of liver glycogen (McCormick and Touster, 1957), D-ribose being also phosphorylated to the 5-phosphate by a liver kinase (Agranoff and Brady, 1956).

The pentose phosphate pathway

The metabolism via pentose phosphates appears to be the main alternative to the glycolytic pathway of carbohydrate metabolism in animal tissues, although quantitatively it is nearly always much less extensive. The broad outline of this route is compared with that of glycolysis in Fig. 1, which shows that there are two points—G6P and F6P—at which these pathways diverge.

(a) The HMP oxidative pathway

G6P is the branching point for this metabolic route, which is also known as the phosphogluconate or oxidative pentose

phosphate pathway. At this stage, G6P can undergo a two-stage TPN-linked oxidation, the first oxidation yielding 6-phosphogluconolactone (Cori and Lipmann, 1952):

$$G6P^{2-} + TPN^{+} \rightarrow 6\text{-PG lactone}^{2-} + TPNH + H^{+}$$

whilst the slow hydrolysis of this lactone is then accelerated by a widely distributed but little studied lactonase (see

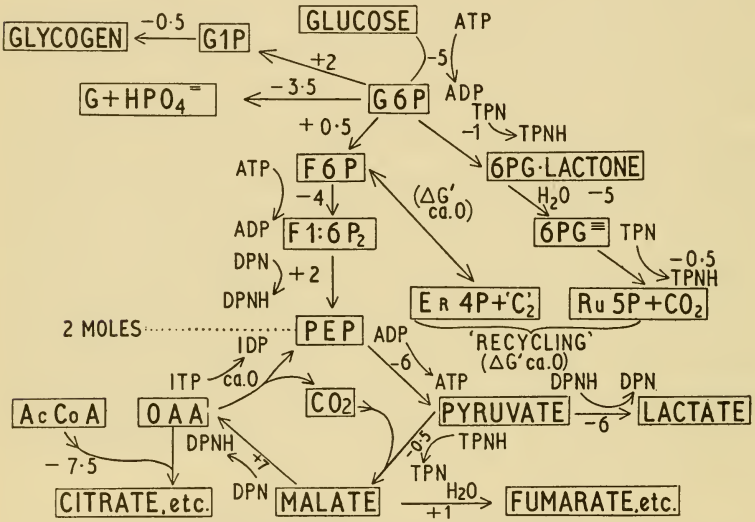


Fig. 2. Approximate free-energy changes involved in glycolysis and the hexose monophosphate oxidative pathways.
Values of ΔG′ represent free energy changes at pH 7 and are rounded to nearest 0·5 kg. cal./mole (Krebs and Kornberg, 1957; see also text). "Citrate, etc" and "Fumarate, etc" indicate points of linking with Krebs cycle reactions.

Fig. 2); the same enzyme appears to hydrolyse δ-gluconolactone (Lipmann and Brodie, 1955):

$$6\text{-PG lactone}^{2-} + H_2O \rightarrow 6\text{-PG}^{3-} + H^{+}.$$

It is unfortunate that accurate free energy values for these reactions are not available. The similar reaction with partially

purified glucose dehydrogenase preparations has given two different values of the equilibrium constant (at 21°):

$$\frac{(\text{Gluconolactone})\,(\text{DPNH})\,(\text{H}^+)}{(\text{Glucose})\,(\text{DPN}^+)} = 3{\cdot}1 \times 10^{-7} \text{ or } 30 \times 10^{-7},$$

respectively (Brink, 1953; Strecker and Korkes, 1952, recalculated, Brink, 1953). These correspond with $\Delta G'$ values (pH constant = 7) of this reaction of $-0{\cdot}7$ or -2 kg. cal., respectively. From the values cited by Krebs and Kornberg (1957), $\Delta G'$ for the hydrolysis and neutralization of the lactone is about -5 to $-6{\cdot}3$. It is assumed in Fig. 2 that the lower values are the more reliable, and that they may be applied to the phosphorylated substrates and to the use of TPN instead of DPN, with either of which coenzymes Brink (1953) reports equal reaction rates (at pH 7·6). On this evidence, the neutralization of the acid would represent the difficultly reversible stage in the HMP oxidative pathway.

For the second TPN oxidative stage:

$$\text{6-PG}^{3-} + \text{TPN}^+ \rightarrow \text{Ru5P}^{2-} + \text{CO}_2 + \text{TPNH}$$

the equilibrium constant, K, has been estimated by Horecker and Smyrniotis (1952) to be quite close to that of the similar *iso*citric dehydrogenase reaction, namely

$$K = \frac{(\text{6-PG})\,(\text{TPN}^+)}{(\text{Ru5P})\,(\text{CO}_2)\,(\text{TPNH})} = 1{\cdot}9 \text{ (moles/l.)}^{-1}.$$

From this the $\Delta G'$ at pH 7 is approximately $-0{\cdot}4$ kg. cal./mole. These values of $\Delta G'$ are used in Fig. 2, which also gives for comparison the approximate free energy changes of related reactions. Those for the glycolytic reactions are taken from the excellent review by Krebs and Kornberg (1957), except that values for phosphatase hydrolysis have been added. Despite some uncertainty about these values (Burton and Krebs, 1953), they are probably not very seriously in error.

(b) Anaerobic pentose phosphate pathway

The pentose phosphates formed from Ru5P in the oxidative stages are able to undergo a fascinating series of changes which have been fully reviewed recently (Racker, 1957;

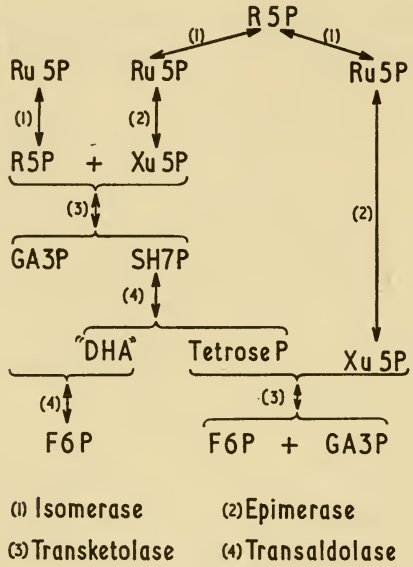


Fig. 3. Anaerobic pentose phosphate cycle.
"DHA" indicates the dihydroxyacetone moiety of SH7P which is transferred to GA3P by TA. Overall reaction: 2 F6P + 1 triose P $\rightleftharpoons$ 3 Pentose P.

Horecker and Hiatt, 1958; Dickens, 1958*b*). The following enzymic reactions occur (cf. Fig. 3):

(1) D-Ru5P $\rightleftharpoons$ D-R5P (ribose phosphate isomerase)

(2) D-Ru5P $\rightleftharpoons$ D-Xu5P (ketopentose phosphate epimerase)

(3) D-Xu5P + D-R5P $\rightleftharpoons$ SH7P + GA3P (TK)

(4) SH7P + GA3P $\rightleftharpoons$ F6P + D-Er4P (TA)

(5) D-Er4P + D-Xu5P $\rightleftharpoons$ F6P + GA3P (TK)

(6) *Overall:* 3 Ru5P $\rightleftharpoons$ 2 F6P + GA3P

Transketolase (TK) requires thiamine pyrophosphate and magnesium ions. Since all of these reactions are quite freely reversible, the individual values of $\Delta G'$ must be quite small, as is indicated in Fig. 2 by "$\Delta G'$ ca. O".

According to Axelrod and Jang (1954), $\Delta G'$ of reaction (1) is $-0{\cdot}7$ kg. cal. (37°, pH 7). There is still considerable doubt about the exact equilibrium positions of these reactions (1)–(5) (see Dickens, 1958*b*; Tabachnick *et al.*, 1958) but the equilibrium constants all appear to lie within the range $0{\cdot}8$–3, i.e. $\Delta G'$ is in the region of zero to unity.

By this method F6P is resynthesized from pentose phosphates, including the D-Xu5P mentioned above as being produced from hexose in the glucuronate cycle, and also from the D-Ru5P arising oxidatively from the TPN-linked dehydrogenases acting as G6P (stage *a*, above). For each turn of the cycle (Figs. 2 and 3), one terminal carbon of hexose is removed as CO_2 and 2 TPN^+ are reduced, one pentose being formed. After six such turns the equivalent of one hexose mole has been completely oxidized to CO_2 and water; the 6 pentose moles formed could then yield, by the sequence of reactions (1)–(5) above, 5 molecules of hexose—one of them being thought to be produced via the action of triose phosphate isomerase, aldolase and fructose diphosphatase, on the GA3P appearing in equation (6) above. During this process no less than 12 moles of TPNH should be produced per mole of hexose disappearing.

Reoxidation of reduced TPN

Whereas the formation of three moles of ATP from ADP is coupled with the oxidation of one mole of DPNH by the flavoprotein-cytochrome oxidative chain of reactions (Lehninger, 1955), a similar proof for the oxidative phosphorylations accompanying TPNH oxidation is lacking at present (Kaplan *et al.*, 1956; Ball and Cooper, 1957).

Joshi, Newburgh and Cheldelin (1957) who used a mitochondrial fraction of heart, supplemented by a soluble fraction,

and Vignais and Vignais (1957) with mitochondria from liver kidney and brain, obtained only very low P/O values of about unity or less for TPNH oxidations. While, therefore, it is not justifiable on present evidence to regard the HMP oxidative pathway as an energy source, there are a number of important biological synthetic systems requiring a supply of TPNH (cf. Horecker and Hiatt, 1958). These include carboxylations and aminations of keto acids, fatty acid and steroid synthesis, and reduction at a physiological pH of dihydrofolic acid to tetrahydrofolic acid, an important stage in one-carbon transfer (Peters and Greenberg, 1958); as well as the reduction of glutathione and various hydroxylations and microsomal detoxication mechanisms (Brodie *et al.*, 1955).

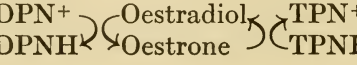
Reoxidation of TPNH can occur (*a*) by the specific TPNH-cytochrome *c* reductase, a flavoprotein system occurring in mitochondria together with (*b*) pyridine nucleotide transhydrogenase (Vignais and Vignais, 1957), the latter being weak in liver and brain mitochondria (cf. Navazio, Ernster and Ernster, 1957). (*c*) Glutathione reductase. (*d*) Two consecutive dehydrogenase reactions each involving one coenzyme e.g. (Holzer and Schneider, 1958):

(i) $\mathrm{TPNH} + \mathrm{H^+} + \alpha\text{-ketoglutarate} + \mathrm{NH_4^+} \rightleftharpoons \mathrm{glutamate} + \mathrm{TPN^+} + \mathrm{H_2O}$

(ii) $\mathrm{Glutamate} + \mathrm{DPN^+} + \mathrm{H_2O} \rightleftharpoons \alpha\text{-ketoglutarate} + \mathrm{DPNH} + \mathrm{H^+} + \mathrm{NH_4^+}$

Sum: $\mathrm{TPNH} + \mathrm{DPN^+} \rightleftharpoons \mathrm{DPNH} + \mathrm{TPN^+}$.

(*e*) Another variant is lactic dehydrogenase, which can react with either coenzyme (Navazio, Ernster and Ernster, 1957; Holzer and Schneider, 1958). (*f*) An ingenious coenzyme-like rôle of steroid hormones acting together with placental 17-β-hydroxysteroid dehydrogenase (Talalay and Williams-Ashman, 1958) is as follows:



Such mechanisms can couple TPN and DPN-specific enzymes, although they have not as yet been much studied from a functional aspect. In liver, the direct reoxidation of TPNH is very slow in comparison with that of DPNH, and in all tissues studied TPNH greatly predominates over TPN^+ (see below).

One of the most important functions of TPNH appears to be in reversal of oxidative decarboxylations. Fig. 4 shows the "by-pass" of the energetically difficult phosphorylation of pyruvate to PEP, the importance of which in resynthesis of carbohydrate from lactate or pyruvate has been emphasized by Krebs and Kornberg (1957). This reaction ($\Delta G'$ about $+ 6$) is believed to be circumvented in liver as shown in Fig. 4 via reductive carboxylation of pyruvate to malate by the TPNH-linked "malic" enzyme, malate oxidation by DPN^+ with malic dehydrogenase to give OAA, and phosphorylative decarboxylation of the latter to give PEP by the reaction studied by Utter and Karahashi (1954):

$$OAA + ITP \rightleftharpoons PEP + CO_2 + IDP$$

Here, the strongly endergonic reaction with DPN^+ (Fig. 4) can be coupled with very efficient reoxidation of the DPNH formed, thus driving the reaction successfully towards OAA.

For this "by-pass" to progress, a continuous supply of TPNH has to be available. Fig. 4 illustrates how the HMP oxidative pathway could be successfully coupled with the "malic" enzyme stage, and the pentose phosphate formed from this oxidation of G6P could be resynthesized to hexose by the TK-TA sequence already described:

(1) $6(G6P^{2-} + 2TPN^+ + H_2O \rightarrow \text{Pentose-}P^{2-} + CO_2 + 2\ TPNH + 2H^+)$

(2) $6\ \text{pentose-}P^{2-} + H_2O \rightarrow 5\ G6P^{2-} + HPO_4^{2-}$

Sum: $G6P^{2-} + 12\ TPN^+ + 7H_2O \rightarrow 6CO_2 + 12\ TPNH + 12H^+ + HPO_4^{2-}$

The 12 TPNH so formed could carboxylate 12 moles of pyruvate:

$$\text{Pyruvate}^- + \text{TPNH} + CO_2 \rightleftharpoons \text{malate}^{2-} + \text{TPN}^+$$

thus yielding, by the "by-pass", 12 PEP and so eventually 6 moles of glucose: ratio of glucose resynthesized/glucose oxidized = 6/1.

The possibility of this coupling of a TPNH-dependent

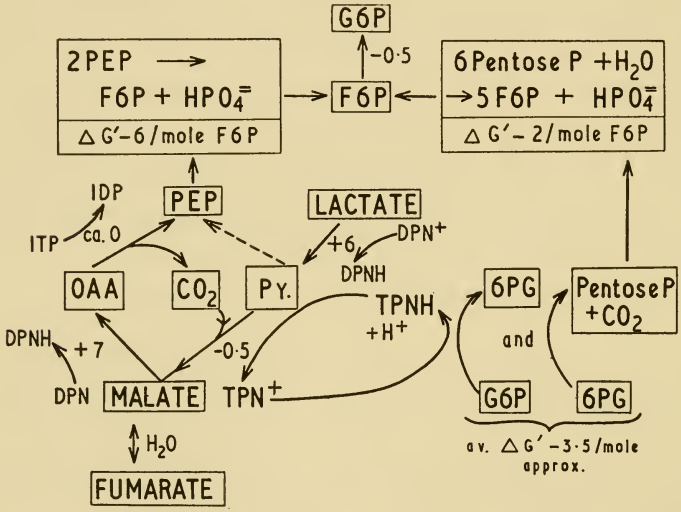


Fig. 4. Coupling of HMP oxidative pathway with carboxylation of pyruvate by means of TPNH.

The numbers on arrows indicate approximate value of $\Delta G'$ for the reactions shown (cf. Fig. 2).

reductive carboxylation with reversal of glycolytic reactions requires the presence of G6P- and 6-PG-dehydrogenases as well as TPN in the tissue concerned. This is the case in liver, a tissue in which randomization of pyruvate carbon atoms in the glucose of the glycogen synthesized accords well with an indirect route of pyruvate incorporation, in which carboxylation to give a stage involving a symmetrical dicarboxylic acid

is followed by decarboxylation; e.g. as indicated in Fig. 2, where rapid fumarase action could produce labelling of both C-2 and C-3 of malate and oxaloacetate. For example [2-^{14}C]-pyruvate would then yield glucose labelled not only at C-2 and C-5 but also at C-1 and C-2 as, in fact, occurs in liver.

What, however, would occur in a tissue like muscle which is very low in its content not only of G6P- and 6-PG-dehydrogenases (Dickens and Glock, 1951; Glock and McLean, 1954) but also has very little total TPN (Glock and McLean, 1955*a*)? The answer has very recently been supplied by Hiatt and co-workers (1958). In rat diaphragm, [2-^{14}C]pyruvate yields on aerobic incubation glycogen labelled almost exclusively in glucose carbons 2 and 5 (39 per cent and 55 per cent of total ^{14}C, respectively). The conclusion seems inescapable that a different mechanism operates in muscle from that which predominates in liver, and the results point to a direct type of incorporation of pyruvate, suggested by Hiatt and co-workers (1958) to be by reversal of the phosphopyruvate kinase reaction. If this actually occurs, there must be a flaw in the reasoning of Krebs and Kornberg (1957, p. 251) that impossibly high ratios of ATP/ADP would be thermodynamically necessary for such a direct reversal.

One possible way out of this dilemma would be the "compartmentalization"* of this part of the ATP. Alternatively, pyruvate could be converted to PEP by an oxido-reduction mechanism. Such a reaction might be, e.g., the oxidation of pyruvate to hydroxypyruvate (or phosphohydroxypyruvate, in presence of ATP) with subsequent reduction to D-glycerate (or phosphoglycerate) which could then enter the glycolytic chain. It could be "resynthesized" to glucose or could presumably, via PEP, be carboxylated to OAA. Small quantities of hydroxypyruvate are present in animal tissues according to Linko and Virtanen (1955) and Holzer and Holldorf (1957). An active glyceric acid kinase is known to be present in liver,

* [At the suggestion of members of the symposium, the word "compartmentalization" is replaced by "compartmentation" throughout this volume. —Eds.]

but as yet no hydroxypyruvate kinase or oxidase has been reported. However, [2-^{14}C]- and [3-^{14}C]hydroxypyruvates are in fact quite well incorporated into liver glycogen, with less randomization of radioactivity in glucose carbons than that reported for pyruvate (Dickens, 1958*a*; Dickens and Williamson, 1959). The incorporation of hydroxypyruvate into muscle glycogen is now being studied, and may provide some evidence as to the possible existence of such a pathway. It is not yet excluded that the incorporation observed in liver may depend on TK acting on hydroxypyruvate and "formaldehyde" (Dickens and Williamson, 1958).

Krebs and Kornberg (1957) reject, on the basis of the earlier experiments of Krebs (1954), the view that in liver G6P- and 6-PG-dehydrogenases could provide enough TPNH for the "malic" enzyme in the above by-pass (cf. Fig. 2). However, the experiments quoted were made with dilute (1 : 30 pigeon liver) homogenates, in which the enzymes themselves and the concentration of total TPN (apparently none was added) must have been very low compared with those in the intact tissue. Their finding that more malate was formed under conditions where ATP was being actively synthesized (aerobic; no DNP added) could perhaps be explained if the possibility of a "direct" type of PEP formation, based on a high ATP level under oxidative conditions, cannot be excluded in these experiments even in the case of liver tissue. This would avoid the necessity for the ingenious explanation advanced by Krebs and Kornberg (1957), that a high ATP level can cause a reduction of TPN^+ to TPNH by a kind of reverse oxidative phosphorylation reaction of the following type:

$$\text{Reduced flavoprotein} + ATP^{4-} + TPN^+ + H_2O \rightarrow \text{flavoprotein} + ADP^{3-} + TPNH + 2H^+ + HPO_4^{2-}.$$

As was pointed out above, such a reaction even in the usual direction of oxidative phosphorylation has not yet been clearly experimentally demonstrated for TPN, although the idea itself is undoubtedly of great theoretical interest.

Data on enzymes concerned with G6P metabolism

Among the factors controlling the relative activities of the glycolytic and pentose P pathways are the enzyme activities both of these pathways and of competing ones, Michaelis constants, substrate concentrations and the presence or absence of inhibitors. Table I shows the activities in liver tissue and some of the characteristics of the two dehydrogenases of the pentose P pathways and of competing enzymes. Although the maximal activities, measured in homogenates or soluble fractions of homogenates under optimal conditions, do not necessarily reflect what is happening in the cell, the relatively low activities of G6P- and 6-PG-dehydrogenases indicate that only a small proportion of the total G6P metabolism proceeds via the pentose P pathway. However, since the Michaelis constants for both substrate and TPN are very low, it is possible that this pathway would compete more efficiently for G6P when the substrate concentration is limiting. The effect of lactonase on the dehydrogenation product of G6P will be to promote this reaction in the forward direction.

Interesting relationships have been demonstrated between the rate of formation of certain intermediates in the pentose P pathway and the activity of enzymes of alternative routes. Thus, 6-PG has been shown to have a marked inhibitory action on phosphoglucose isomerase when present in the same molecular concentration as G6P or F6P (Parr, 1956). In addition, the rate of glycogen synthesis is modified by accumulation of R5P since this inhibits phosphoglucomatase activity (Segal and Foley, 1958). A more direct effect is the inhibition of brain hexokinase activity by G6P, the product of the reaction (Weil-Malherbe and Bone, 1951; Crane and Sols, 1953). It is possible that the high concentrations of G6P in some tissues (Weil-Malherbe, 1955) may regulate to some extent the activity of this enzyme.

Table I

Characteristics and activities of enzymes concerned with G6P metabolism

	Phosphoglucose isomerase	*Phosphoglucomutase*	*G6P dehydrogenase*	*6-PG dehydrogenase*	*G6Pase*
μmoles G6P metabolized per g. liver per hr. at 37°	7,600	4,950	115	124	1,100
Michaelis constants					
K_S	$1{\cdot}7 \times 10^{-3}$ M[a]		$1{\cdot}3 \times 10^{-5}$ M[b]	$1{\cdot}0 \times 10^{-5}$ M[b]	5×10^{-3} M[c]
K_{TPN}			$1{\cdot}3 \times 10^{-5}$ M[b]	$2{\cdot}8 \times 10^{-5}$ M[b]	
$K_{G1:6P_2}$		5×10^{-7} M[d]			
$\Delta G'$ (kg. cal./mole)	+0·5	+1·7	−5·5 (approx.)†	−0·5 (approx.)	−3·5 (approx.)
Equilibrium position	$\frac{\text{F-6-P}}{\text{G-6-P}} = \frac{30}{70}$	$\frac{\text{G 1-P}}{\text{G 6-P}} = \frac{5}{95}$	—	$\frac{65}{35}$*[e]	

* (Ru 5-P) (CO_2) (TPNH)/(6-PG) (TPN^+).

† Including lactonase reaction.

a Tsuboi, Estrada and Hudson (1958); *b* Glock and McLean (1953); *c* Beaufay and de Duve (1954); *d* Sutherland *et al.* (1949); *e* Horecker and Smyrniotis (1952).

Determination of the relative importance of the pentose phosphate and glycolytic pathways

Various isotope methods have been devised for determining the relative contributions of the pentose P and glycolytic pathways to the total glucose catabolism of tissues involving the use of glucose specifically labelled with ^{14}C in one or more positions. Of these, the method most widely employed is to determine the incorporation of C-1 and C-6 from [1-^{14}C]- and [6-^{14}C]-glucose into CO_2. In others, the incorporation of ^{14}C into, e.g., lactate, fatty acids and acetoacetate is measured. The validity of all these methods depends on two major assumptions, firstly that recycling of hexose is not extensive and secondly that there is rapid equilibration of triose phosphates by triose phosphate isomerase. One of the important differences between these two types of method is that in the latter the end product is formed after only relatively few enzyme reactions. There are thus fewer opportunities for isotope dilution and loss of isotope by side reactions than when oxidation occurs via the complete glycolytic-tricarboxylic acid sequence of events.

Estimates of the relative contribution of the pentose P and glycolytic pathways to the glucose metabolism of liver by these two routes calculated from four different isotope methods are shown in Table II. Although quantitative evaluation is difficult and open to criticism (Wood, 1955) the results for liver slices are in fairly good agreement and indicate that 10–25 per cent of the glucose catabolized *to the particular end product* being measured arises from the pentose P pathway and 75–90 per cent from the glycolytic route. A higher contribution of the pentose P pathway (an average of approximately 40 per cent) was observed in experiments with perfused rat liver (Murphy and Muntz, 1957) and after intraportal injection of labelled glucose (Muntz and Murphy, 1957). This can probably be attributed to the fact that, in the former experiments, the liver was perfused with a constant physiological concentration of glucose since the blood was not

Table II

Participation of pentose phosphate and glycolytic pathways in glucose catabolism in liver, calculated from experiments using specifically labelled glucose

		Relative Contribution [Percentage]		
	Product measured	*Pentose P pathway*	*Glycolytic route*	*References*
Liver slices	CO_2	10	90	*a*
	Lactate	12	88	*b*
	Fatty Acids	14	86	*c*
	Acetoacetate	25	75	*d*
Perfused liver	CO_2	56	44	*e*
Liver *in vivo*	Lactate	35	65	*f*

a Katz *et al.* (1954).
b Ashmore *et al.* (1956).
c Bloom and Stetten (1955).
d Wenner and Weinhouse (1956*a*).
e Murphy and Muntz (1957).
f Muntz and Murphy (1957).

recirculated, and in the latter experiments to the fact that they were of only a few seconds' duration so that recycling was reduced to a minimum.

Although this paper is concerned with oxidative pathways of glucose metabolism, it is important to realize that they account for only a part, and under certain conditions for only a small part, of the total G6P metabolized by liver. In this connexion, the recent work of Hastings, Ashmore and associates is of great importance. Employing differentially labelled glucose and uniformly labelled fructose, Ashmore and coworkers (1957) measured the uptake of sugars and their conversion to glucose, glycogen and CO_2 in liver slices under different conditions. From these results they calculated the amounts of G6P metabolized by the four alternative pathways. Some of their results are summarized in Fig. 5, from which it is obvious that the pattern of metabolism is altered considerably both by changes in cation concentrations of the suspending medium and more profoundly in liver slices from diabetic rats. Glycogen synthesis is much greater in a high K^+ medium than in a high Na^+ medium. This enhanced glycogen synthesis is mainly at the expense of the E-M

pathway since the other two pathways are scarcely affected. The lower glycogen synthesis in a high Na^+ medium is due to loss of intracellular K^+ and replacement by Na^+ which has been shown to alter the activation-inactivation mechanism in favour of increased phosphorylase activity (Cahill *et al.*,

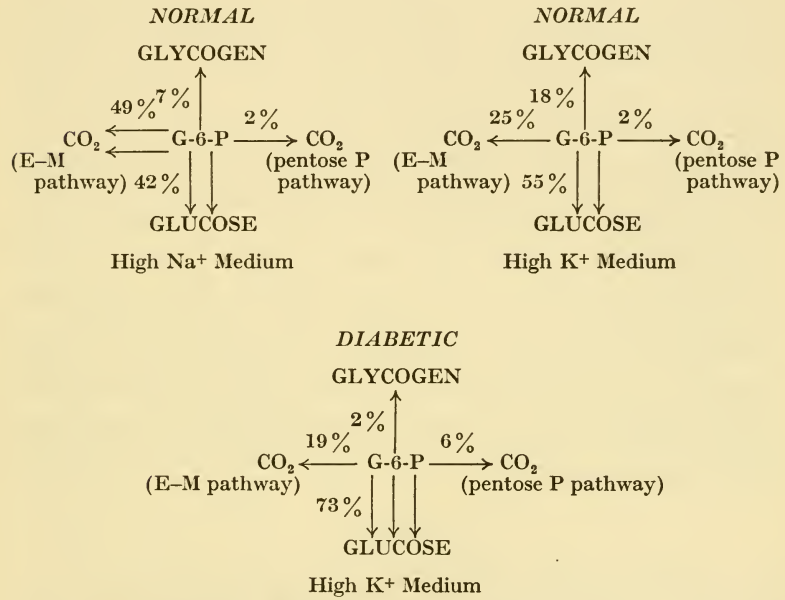


Fig. 5. Pathways of G6P metabolism in rat liver slices. Data of Ashmore and co-workers (1957).

1957). Glucagon and adrenaline produce a similar effect (Cahill *et al.*, 1957; Berthet, Sutherland and de Duve, 1957). In diabetes the ability to phosphorylate glucose is very greatly reduced and Ashmore and co-workers (1957) found that liver slices only phosphorylated 10 μmoles glucose per g. liver in 90 minutes in comparison with normal liver slices which phosphorylated about four times this amount. Of the G6P available, a much smaller proportion is used for glycogen synthesis. This is probably related to increased G6Pase

activity. The proportion of G6P metabolized by the glycolytic route is not greatly affected, although there is a significant increase in the small proportion oxidized by the pentose P pathway. These findings emphasize the magnitude of G6Pase activity in liver and its importance in controlling pathways of glucose metabolism. The effect of various hormonal treatments and of tumour formation on the activity of the pentose P pathway has been assessed both by determination of individual enzyme activities and also by the use of labelled glucose. Although C-1/C-6 quotients are of doubtful quantitative significance, alterations in this quotient in any one tissue from animals under different physiological conditions gives some qualitative indication of the relative activities of the glycolytic and pentose P pathways. Unfortunately, results obtained with the isotope method do not always corroborate findings on enzyme activities, thus emphasizing the necessity for determining the overall activities of metabolic pathways in addition to the activities of single enzymes. Results obtained for liver from normal, diabetic and hyperthyroid rats and for precancerous liver tissue, DAB-induced hepatomata and embryonic liver are given in Fig. 6 and Table III.

Table III

Yields of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose by liver and liver tumour slices from rats

	Percentage conversion to $^{14}CO_2$		
	[1-^{14}C]*glucose*	[6-^{14}C]*glucose*	C-1/C-6
Control	2·0	1·2	1·7
Alloxan diabetic			
No insulin	1·5	0·5	3·0
Insulin	3·0	1·3	2·3
Control	1·5	0·8	1·9
Hyperthyroid	1·7	1·3	1·3
Control	5·0	1·6	3·1
DAB liver tumours	4·5	2·8	1·6

Data from Agranoff, Brady and Colodzin (1954); Glock, McLean and Whitehead (1956).

Hormonal effects on oxidative pathways in liver

Although the activity of G6P dehydrogenase is not markedly changed in the liver of diabetic rats, that of 6-PG dehydrogenase falls to about half the control value. The slight depression in the rate of formation of CO_2 from C-1 of glucose (Table III), may be related to the low Michaelis constants of these enzymes which results in the oxidation of a relatively large part of the available G6P by this route. This effect is reflected in the high C-1/C-6 quotient observed in liver slices from diabetic rats. Treatment of the diabetic animals with insulin produced a significant increase in the activity of G6P dehydrogenase and 6-PG dehydrogenase (Glock, McLean and Whitehead, 1956). This was accompanied by a significant rise in the yield of $^{14}CO_2$ from [1-^{14}C]glucose, above both diabetic and control levels. The yield of $^{14}CO_2$ from [6-^{14}C]-glucose was increased even more by insulin treatment, resulting in a return of the C-1/C-6 quotient towards the control value (Table III).

Chaikoff (1953) has postulated two blocks in the metabolism of glucose in the diabetic animal, one at the stage of glucose phosphorylation and the other at the level of the 2-carbon oxidative fragment. The above results using differentially labelled glucose molecules are in accord with this.

The results obtained with isotopes for liver slices from diabetic rats do not agree with those of Bloom (1955) who found a lowered value for the C-1/C-6 quotient, indicating that a greater proportion of glucose was being catabolized via the glycolytic route. These discrepancies may perhaps be attributed to the fact that in the experiments of Bloom the diabetes was more severe and of longer duration.

In the case of liver slices from thyroxine-treated rats, the isotope results indicate that the increased glucose catabolism is due to a greater participation of the glycolytic pathway, since the yield of $^{14}CO_2$ from [6-^{14}C]glucose only is increased, and there is a significant fall in the C-1/C-6 quotient from 1·9 in the control group to 1·3 in the hyperthyroid group. It thus appears that despite the increased activity of enzymes of

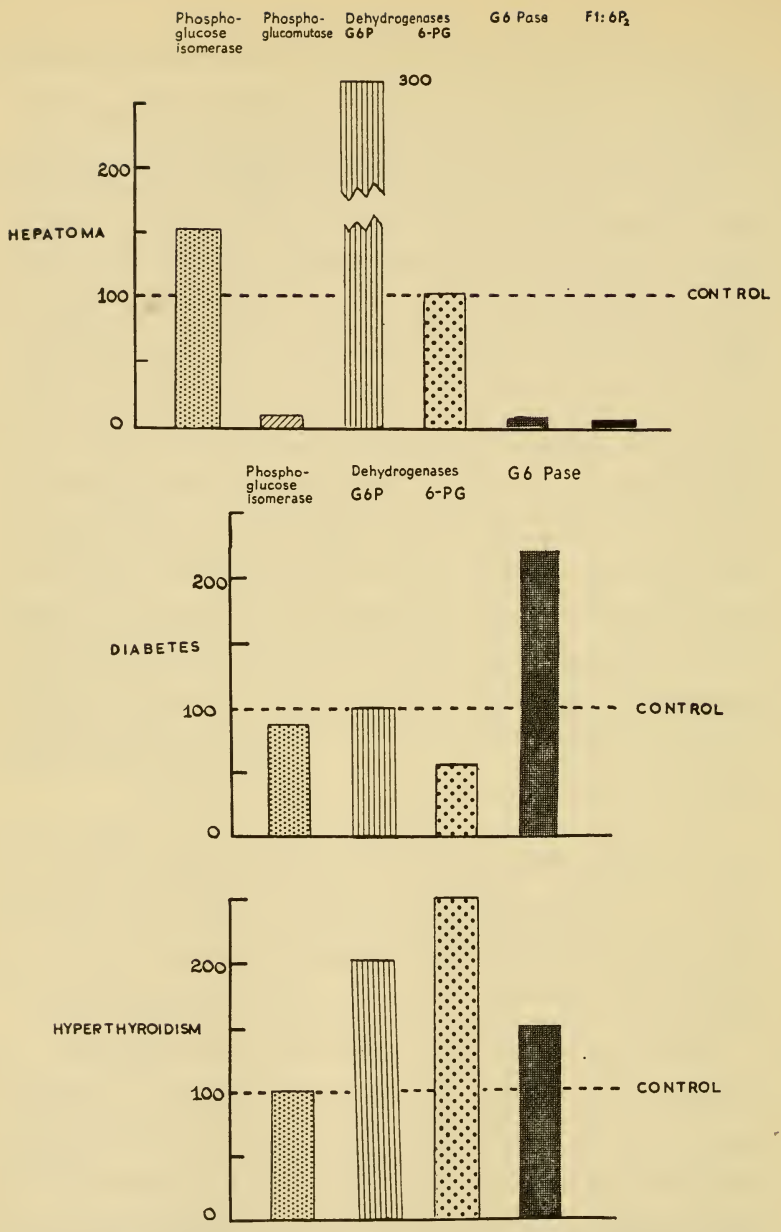


FIG. 6. Levels of activities of various enzymes connected with G6P metabolism in livers of diabetic and hyperthyroid rats and in hepatomata. Composite data from Ashmore and co-workers (1954); Glock, McLean and Whitehead (1956); Weber and Ashmore (1958), and Weber and Cantero (1957). $F1:6P_2$=fructose-1;6-diphosphatase.

the pentose P pathway in the hyperthyroid rat liver, the glycolytic pathway competes more effectively for the available substrate. It is interesting to compare these results with those obtained by Spiro and Ball (1958) who made a comparison of the total and radioactive CO_2 expired by normal and hyperthyroid rats after injection of [1-^{14}C]glucose and [6-^{14}C]glucose. Except for the first 15-minute period after injection of the labelled glucose, the C-1/C-6 quotient was found to be the same in the hyperthyroid as in the normal animals, the average value for this quotient being 1·4. These authors interpret this to mean that the enhanced metabolic rate observed in the hyperthyroid rat reflects an approximately equal increase in the amounts of glucose metabolized by the glycolytic and pentose P routes.

The increased G6Pase activity shown in Fig. 6 may perhaps be correlated with the low glycogen content of liver from hyperthyroid rats (Cramer and Krause, 1913). Olsen (1951) has stated that in rat liver the step between F6P and F1:6P_2 is rate-limiting in the sequence of reactions between G6P and lactate. It would be of considerable interest to know the activity of this enzyme in relation to those of the hexose-monophosphate oxidative route in these hormonal conditions.

Oxidative pathways in liver tumours

Wenner and Weinhouse (1956*a*) have calculated, from data obtained on the incorporation of ^{14}C from labelled glucose into lactic acid in a series of tumours, that the non-glycolytic pathway could account for only a small part (from 0–16 per cent) of the total glucose catabolized. That the pentose P pathway does, however, operate in tumours is indicated from experiments employing [1-^{14}C]glucose and [6-^{14}C]glucose. A preferential conversion of C-1 of glucose to CO_2 by various types of liver tumours has been demonstrated by Abraham, Hill and Chaikoff (1955), Emmelot (1955) and Wenner and Weinhouse (1956*a*). The C-1/C-6 quotient of slices of liver and liver tumours from rats fed DAB (Agranoff, Brady and Colodzin, 1954) are shown in Table III. These results suggest

that in these liver tumours, at any rate, a smaller proportion of the respiratory CO_2 is derived from the pentose P pathway than in normal liver.

Some very striking alterations in the pattern of enzyme activity, particularly among enzymes concerned with G6P metabolism, have been demonstrated in liver tumours. Weber and Cantero (1957) have shown that the activity of G6P dehydrogenase in Novikoff hepatoma is five times higher than that of normal rat liver. A similar increase in the G6P dehydrogenase activity has been found in the liver tumours of DAB-treated rats while, in marked contrast to this, the activity of 6-PG dehydrogenase remained unchanged and thus becomes the limiting enzyme (Glock and McLean, 1958, unpublished). The reduced overall activity of this pathway cannot, therefore, be explained by these alterations in enzyme activity and it is perhaps due to the very low TPN content of the tumours, which may become rate-limiting (Glock and McLean, 1957). The supply of TPN^+ may be even further limited by their very low levels of TPNH-cytochrome *c* reductase and pyridine nucleotide transhydrogenase (Reinafarje and Potter, 1957).

There is a progressive decrease in the G6Pase activity of liver which occurs quite early during the induction of DAB tumours (Spain, 1956) and in certain liver tumours no G6Pase activity could be detected (Weber and Cantero, 1957). Another function of the normal liver cell which is lost in neoplastic tissue is the storage of glycogen. The related enzymic changes are particularly clearly seen in Novikoff hepatoma where there is a very marked decrease in phosphoglucomutase activity (to less than 10 per cent of that in normal liver) and a complete absence of fructose diphosphatase, an enzyme of importance in the synthesis of glucose from pyruvate and lactate (Weber and Cantero, 1957).

Synthesis of ribose

The two alternative pathways for ribose synthesis can be assessed *in vivo* by measurements of the incorporation of ^{14}C

from [2-^{14}C]glucose into either visceral RNA ribose or into ribose isolated from the riboside excreted after administration of imidazole acetic acid. Ribose synthesized via the oxidative loss of C-1 of [2-^{14}C]glucose would be labelled in its first carbon atom while that produced by non-oxidative transfer of the first two carbon atoms of hexose phosphate to a triose phosphate acceptor, as catalysed by TK, would be labelled in C-2. The C-1/C-2 quotient gives an indication of the relative contribution of the two pathways to ribose synthesis. A low C-1/C-2 ratio is consistent with the operation of the non-oxidative TK pathway whereas a high ratio indicates the predominance of oxidative decarboxylation. Some results obtained by the use of these methods (Hiatt, 1957, 1958; Feigelson and Marks, 1957) are shown in Table IV.

Table IV

Incorporation of ^{14}C from [2-^{14}C]glucose into C-1 and C-2 of ribose (expressed as C-1/C-2)

Condition	*Excreted riboside**	*Ribose of liver RNA*
Normal	0·5–0·8	0·7
Bearing ascites tumour cells	0·8–1·4	0·9–1·0
Hyperthyroid	0·5–0·8	
Thiamine-deficient	2·6	

* After administration of imidazoleacetic acid to rats.

Composite data from Hiatt (1957, 1958) and Feigelson and Marks (1957).

The C-1/C-2 quotient is unaltered in hyperthyroidism, increased somewhat in rats bearing ascites tumour cells and markedly increased in thiamine-deficient rats, this latter effect probably being attributable to a lower TK activity in the absence of its essential cofactor, thiamine pyrophosphate.

Levels of coenzymes

Total tissue levels of DPN ($DPN^+ + DPNH$) and TPN ($TPN^+ + TPNH$) as well as the relative proportion of oxidized

and reduced coenzymes would be expected to play an important part in the regulation of alternative pathways of carbohydrate metabolism. In addition, certain metabolic derangements such as occur, for example, in diabetes and tumour formation might produce alterations in the steady-state concentrations of the oxidized and reduced forms. Table V incorporates results for liver from normal and alloxan diabetic rats, from rats rendered hyperthyroid by thyroxine injections (Glock and McLean, 1955*b*) and for liver tumours induced in DAB feeding (Glock and McLean, 1957).

Table V

Levels of oxidized and reduced coenzymes in liver and liver tumours of rats

	DPN$^+$ μg./g.	*DPNH* μg./g.	*DPN*$^+$/*DPNH*	*TPN*$^+$ μg./g.	*TPNH* μg./g.
Control	407	161	2·7	10	148
Diabetic	324	217	1·5	8	158
Control	446	158	2·9	4	206
Hyperthyroid	393	164	2·4	2	155
Control*	441	157	2·9	4	92
DAB liver tumour	226	83	2·7	3	27

* Tissue adjacent to liver tumour.

The DPN$^+$/DPNH quotient is significantly lowered in diabetes, due both to a fall in DPN$^+$ and an increase in DPNH. This lowered quotient may perhaps be a result of decreased lipogenesis. However, on account of the multiplicity of coenzyme-linked reactions and our present very incomplete knowledge as to how these may be affected in different pathological conditions, it appears unwarranted to speculate as to the exact cause of any observed change in this quotient. The total TPN content per g. liver is not affected in diabetes, but total DPN and TPN are both significantly reduced if expressed on a whole liver basis. The above findings on altered DPN$^+$/DPNH$^+$ quotients do not agree with those of Helmreich and co-workers (1954) and Greenbaum and Graymore (1956). These two groups of workers, using the same

method of estimation, found increased DPN^+/DPNH quotients, due chiefly to a decrease in DPNH. They attributed this to deranged glycolysis which would result in decreased availability of DPNH and consequently depressed lipogenesis (Helmreich *et al*, 1954; Brand and Helmreich, 1956). It is difficult to account for these discrepancies, but they may be related to the degree of diabetes in the animals.

The only marked effect in the hyperthyroid animals is on the total TPN (principally TPNH) content of liver which is decreased both on a unit weight basis and especially on a whole liver basis. There is also a decrease in both DPN^+ and total DPN on a whole liver basis. The coenzyme contents of diaphragm are not affected. It is possible that these reduced coenzyme levels in liver in hyperthyroidism may be related to decreased mitochondrial synthesis of ATP due to uncoupling of oxidative phosphorylation. Uncoupling by thyroxine has been demonstrated both on addition of thyroxine *in vitro* (Lardy and Maley, 1954) and in mitochondrial preparations from hyperthyroid animals (Hoch and Lipmann, 1953; Martius, 1956). It should be emphasized, however, that these effects have only been demonstrated with large and unphysiological concentrations of thyroxine. Recent experiments by Bronk (1958) have indeed shown that the primary action of thyroxine on isolated mitochondria is to increase the efficiency of oxidative phosphorylation. Uncoupling only occurs with thyroxine if mitochondria are preincubated in the absence of substrate and thyroxine, although Dickens and Salmony (1956) observed an immediate action of thyroacetic acids. Moreover, the uncoupling which occurs in isolated rat liver mitochondria in hypotonic sucrose and in mitochondria from hyperthyroid rats can be completely reversed by sufficient Mg^{2+}, implying a primary effect of thyroxine on mitochondrial permeability (Tapley and Cooper, 1956). Recent results of Emmelot and Bos (1958) also suggest that thyroxine modifies the functional integrity of mitochondria rather than that it has a direct effect on phosphorylation.

Reduced liver coenzyme levels in hyperthyroidism cannot

be explained by decreased DPN pyrophosphorylase activity (Glock and McLean, 1958, unpublished). DPNase or TPNase activity might be increased, but this requires investigating. It is of interest that the activities of two of the enzymes concerned with the reoxidation of TPNH are altered by thyroxine. TPNH cytochrome *c* reductase activity is approximately doubled in the liver of hyperthyroid rats (Phillips and Langdon, 1956) whereas thyroxine inhibits the *in vitro* oxidation of TPNH by transhydrogenase (Ball and Cooper, 1957). Inhibition of transhydrogenase activity, if it occurs *in vivo*, might perhaps be expected to result in a lower yield of high-energy phosphate.

The total DPN content of DAB hepatomata is approximately half that of normal liver. The DPN^+/DPNH quotient, however, is unaltered. The total DPN contents of a large range of tumours are very similar to those quoted in Table V (Jedeikin and Weinhouse, 1955; Glock and McLean, 1957) and in all cases the DPN^+/DPNH quotient is not significantly different from that of normal tissues (Glock and McLean, 1955*a*). The unaltered DPN^+/DPNH quotient is somewhat surprising in view of the high lactic acid concentrations in many tumours which would be expected to be reflected in this quotient. Of more interest is the finding that the total TPN (chiefly TPNH) content of all the tumours investigated is extremely low (Glock and McLean, 1957). As shown in Table V, there is a gradual fall in the total TPN content of rat liver undergoing carcinogenesis with DAB, the TPN content of the liver adjacent to the tumours being less than half that of normal liver.

The relatively low DPN content of tumours cannot be attributed to increased DPNase activity (Quastel and Zatman, 1953) and is presumably due to decreased DPN pyrophosphorylase activity, since Branster and Morton (1956) and Morton (1958) have found a marked reduction in the activity of this enzyme in certain tumours. The results in Table V suggest, in addition, that synthesis of TPN from DPN is also severely depressed in DAB tumours.

Intracellular distribution of coenzymes

The general finding that, in animal tissues and tumours, DPN is present predominantly in the oxidized form whereas TPN is almost exclusively in the reduced form (Glock and McLean, 1955*a*), indicates that these coenzymes are relatively inaccessible to each other. This suggests that they are either largely bound to other cell constituents or located in different cell compartments.

The intracellular distribution of DPN^+, DPNH, TPN^+ and TPNH in rat liver (Glock and McLean, 1956) is shown in Table VI. It is of interest that the total TPN content of liver

Table VI

Intracellular distribution of pyridine nucleotides in rat liver (μg./g.)

	DPN$^+$	*DPNH*	*TPN*$^+$	*TPNH*	*Total PN*
Homogenate	425	116	25	231	797
Nuclei	22	13	5	17	57
Mitochondria	47	21	3	90	161
Microsomes	16	3	4	3	26
Soluble fraction	359	50	26	88	523

mitochondria accounts for approximately 36 per cent of the total cellular TPN and is of the same order of magnitude as the total DPN in the mitochondria. Much more of the total DPN (75 per cent) than of the TPN (45 per cent) is present in the soluble fraction. These results are slightly different from those of Jacobson and Kaplan (1957) who found a smaller proportion of the total DPN in the mitochondria.

This work is now being extended to tumours and to the liver of rats under different hormonal treatments. The intracellular distribution of coenzymes will be of particular interest in those conditions where marked changes in mitochondrial numbers and structure have been demonstrated.

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DISCUSSION

Chance: Prof. Dickens, with reference to your Table VI which showed the DPNH/DPN and TPNH/TPN ratios in mitochondria, did you treat the mitochondria with substrate prior to analysis or did they contain only endogenous substrate?

Dickens: They were not treated with substrate.

Chance: Disruption of the mitochondria during analysis apparently causes some oxidation of reduced pyridine nucleotide by activation of the respiratory chain. Apparently this occurs just as you add perchlorate to stop the reaction. It also appears that the intramitochondrial DPNH can be oxidized without affecting TPNH to the same degree. This may be due to the fact that transhydrogenase activity is rather low, the TPNH has not had time to follow the DPNH.

Dickens: We found low transhydrogenase activities in liver too, but Prof. Slater has stated earlier in this symposium (p. 13) that Dr. Purvis in his laboratory has found reasonably adequate transhydrogenase activity. With regard to the oxidation during the preparation, I suppose that is a possibility. You mean that DPNH would be oxidized preferentially, and the TPNH remain?

Chance: DPNH can be rapidly oxidized by the isolated respiratory chain.

Dickens: It would be very difficult on this basis to explain the fact that when the whole tissue is taken out of the animal and rapidly dropped into boiling acid or alkali, as the case may be, very much the same distribution of oxidized and reduced coenzymes is obtained; i.e. in the whole tissue the total DPN is about 70 per cent or more oxidized, while the TPN is nearly all present as TPNH.

Glock: There is, in fact, about the same proportion of oxidized to reduced form of each of the pyridine nucleotides in the whole tissue as in the isolated mitochondria.

King: In view of the fact that the pentose cycle enzymes exist almost exclusively in the soluble, non-mitochondrial fraction, I wonder whether there is another route for the direct oxidation of TPNH in the soluble fraction.

Dickens: It was first shown by Drs. Glock and McLean that these enzymes are mainly in the soluble fraction of the cell. Reduced TPN is therefore presumably mainly produced by this system in the soluble part, and yet the mitochondria are full of reduced TPN. This is puzzling. As regards the oxidative systems in the soluble part, they must be comparatively inefficient too, because otherwise the whole TPN in the cell would not be mainly in a reduced state, which it is.

de Duve: Some observations made by Dr. Hers in my laboratory seem to confirm the different state of oxidation of the two coenzymes. The system studied by Dr. Hers is the conversion of glucose to fructose in seminal vesicles. This appears to go through sorbitol by way of a reduction which uses TPNH as an electron donor, and a subsequent oxidation which uses DPN as an electron acceptor. In slices of tissue, and presumably also in the intact tissue, the reaction is essentially

irreversible, although the equilibrium between glucose and fructose should, in fact, favour glucose to some extent. Dr. Hers believes that the factor which actually drives the reaction towards fructose is the difference in potential between the two coenzymes. Thus, the reaction may be said to derive its energy from a coupled electron transfer between TPNH and DPN.

A number of systems have now been described which appear to be coupled to the transfer of electrons between TPNH and DPN. A very interesting one is that shown by Prof. Dickens, going from pyruvate to phosphopyruvate via malate and oxaloacetate. The importance of TPNH and DPN in this system has been stressed before by our Chairman. Another one is the L-xylulose to D-xylulose transformation, which goes via xylitol—this has been described by Touster and co-workers (Touster, O., Reynolds, V. H., and Hutcheson, R. M. (1956). *J. biol. Chem.*, **221**, 697). This view, if correct, would give a special significance to the transhydrogenase as being some kind of uncoupling enzyme for these phenomena which depend on the transfer of electrons between TPNH and DPN for their supply of energy.

Dr. Hers has also found that he can increase the activity of the pentose phosphate cycle as measured by the production of $^{14}CO_2$ from [1-^{14}C]-labelled glucose in liver slices, by adding substrates of aldose reductase, the enzyme which will reduce various aldose compounds with the aid of TPNH. Adding substrates of this enzyme to liver slices will increase the operation of the pentose phosphate cycle several fold. Again this is an indication that the reoxidation of TPNH is somehow inhibited in these slices and can be stepped up by adding a substrate which will act as an electron acceptor from TPNH.

Dickens: The glucose-sorbitol-fructose system is, of course, very restricted in that it occurs in prostate and in the seminal vesicles. A number of other systems which can similarly effect a transhydrogenase type of activity, and which occur more widely, are mentioned in the printed account, but time did not permit their inclusion when presenting this paper.

Racker: In *Acetobacter xylinum* there is actually a reaction which yields an energy-rich phosphate associated with the shunt pathway without being directly linked to TPNH oxidation. I am referring to the phosphorolytic cleavage of fructose-6-phosphate to acetyl phosphate and erythrose-4-phosphate. The enzyme which catalyses the reaction has been named fructose-6-phosphate phosphoketolase. The erythrose-4-phosphate which arises from this reaction can cycle back via the pentose phosphate cycle to fructose-6-phosphate. By repetition of the phosphorolytic cleavage of fructose-6-phosphate and recycling of the erythrose-4-phosphate, one can visualize that the entire hexose molecule is cleaved to 3 moles of acetyl phosphate. Since these micro-organisms contain an active acetokinase, they can convert acetyl phosphate + ADP → acetate and ATP. Thus, the overall reaction yields 3 moles of ATP per mole of fructose-6-phosphate or, if one subtracts one ATP required for phosphorylation of glucose, the overall yield is two moles of ATP per mole of glucose, which is identical with the energy yield of

the glycolytic pathway. We discovered this fructose-6-phosphate phosphoketolase enzyme in the course of unsuccessful attempts to demonstrate phosphorylation linked to the oxidation of TPNH.

Dickens: This enzyme has not been described other than in micro-organisms?

Racker: We looked for it in animal tissues but could not find it.

Greville: Is there any evidence, at least in animal tissues, that the pentose phosphate pathway forms a cycle, i.e. that it goes from glucose-6-phosphate through the pentose phosphates and back to glucose-6-phosphate?

Dickens: Yes, I think so. This is quite clear from the work of Marks and others (Hiatt, 1957, 1958, *loc. cit.*; Feigelson and Marks, 1957, *loc. cit.*; Marks, P. A., and Feigelson, P. (1957). *J. biol. Chem.*, **226**, 1001). Labelling of the glycogen glucose and RNA-ribose molecules, derived particularly from glucose labelled in the 2-position, indicates quite clearly that one first-stage reaction is decarboxylation at C–1 of the glucose, and a build-up to hexose. The labelling of the carbons fits the participation of this pathway very well. That is in the intact animal. An extensive non-oxidative transketolase-transaldolase interconversion of hexose and pentose also occurs.

Holzer: You mentioned the discrepancy between our results and those of Glock and McLean concerning the determination of DPNH and DPN in diabetic animals. This might be due to our animals having too much alloxan. We did not attempt to clarify this because, in the meantime, together with Prof. Lynen we carried out experiments on yeast cells (Holzer, H., Holzer, E., and Schultz, G. (1955). *Biochem. Z.*, **326**, 385; Holzer, H., Schultz, G., and Lynen, F. (1956). *Biochem. Z.*, **328**, 252) showing that the concentrations of *free* DPNH and DPN (which are responsible for the thermodynamic situation) are different by a factor of 100 from those measured with the methods used hitherto. In these experiments we determined the concentrations of acetaldehyde and alcohol, and calculated from these data, by means of the equilibrium constant of the alcohol dehydrogenase reaction, the DPNH/DPN ratio. It would be of interest to carry out the same type of experiments with animal tissues, since it is likely that in animal tissues there exists an equilibrium between pyruvate, lactate, DPNH and DPN, catalysed by lactic dehydrogenase which is very active in animal tissues. From the analyses of lactate and pyruvate the ratio of free DPNH to DPN might be calculated (cf. Bücher, Th., and Klingenberg, M. (1958). *Angew. Chem.*, **70**, 552).

Dickens: That would be extremely valuable and certainly needs to be done.

Racker: Prof. Dickens mentioned that the animals had too little alloxan, and Prof. Holzer mentioned that they had too much alloxan—what is the right amount?

Dickens: If you give them an enormous dose, they are in a moribund condition, not only from diabetes, but probably because of other non-specific toxic effects of alloxan.

Holzer: Ours had 700 to 1000 mg. per cent of glucose in the blood.

This is extremely high and, therefore, not only diabetes but also general damage might possibly have occurred.

Hess: In relation to Prof. Holzer's comment and Prof. Dickens' answer on the pyruvate-lactate equilibrium in animal tissue, I might mention that we have already done this type of analysis, and also with similar substrate redox systems, in our laboratory. Dr. Czok, in Prof. Bücher's group, is also engaged in these studies. We applied the method to human blood which was withdrawn under resting conditions and under various pathological conditions. Since lactic dehydrogenase is very strongly concentrated in human tissue (unpublished data), and since there is no diffusion problem for both lactate and pyruvate, the ratio of both substrates measured in venous or arterial blood reflects the overall ratio of the tissue, muscle playing the largest part. Under normal conditions we find a molar lactate/pyruvate ratio of 12·97 corresponding to a substrate redox potential of $E'_h = -222{\cdot}9$ mv. Thus, using Racker's equilibrium constant for lactic dehydrogenase for the calculation of the DPN/DPNH ratio, we obtain a value of 1927. Under hyperglycaemic conditions in normal patients, the lactate/pyruvate ratio is shifted to 19·14 (case no. 2*b*) or 26·5 (case no. 3). Under hyperglycaemic conditions in diabetic patients the ratios were 23·3 (case no. 18) or even 35·6 (case no. 19). The latter value, calculated for the DPN/DPNH ratio, would be 700. This drop in the ratio (Hess, B. and Gottschling, H. (1959). In preparation) is in agreement with the data presented by Prof. Dickens.

Dickens: Is that ratio the same as that of the total amounts of oxidized and reduced pyridine nucleotide actually present in the animal tissues?

Hess: Yes, just the same.

Potter: Prof. Bücher and his co-workers at Marburg are carrying out quite extensive studies on this in terms of lactate and pyruvate, and also in terms of dihydroxyacetone and α-phosphoglycerol.

King: In connexion with Prof. de Duve's comment on the conversion of glucose to fructose via sorbitol, Dr. Mann and I have found that this occurs not only in seminal vesicles, but also in spermatozoa *per se*. We have isolated soluble sorbitol dehydrogenase from spermatozoa and purified it to some extent. We believe that this sorbitol dehydrogenase in spermatozoa acts as a regulatory factor for the various activities which require pyridine nucleotide, since semen contains no glucose, but only fructose. Therefore, by this series of reactions the pyridine nucleotides, oxidized or reduced, can be supplied, depending on the prevalent conditions (e.g. concentrations of reactants) which can certainly affect the direction of this series of reversible reactions.

Prof. Dickens, you indicated that CO_2 came either from the Embden-Meyerhof or from the pentose phosphate pathway. How can you differentiate between the two?

Dickens: We cannot; but by using glucose labelled in positions 1 and 6 and comparing the yields of $^{14}CO_2$ then obtained, one can calculate the probable relative extent of the pathways, on the assumption that the C–1 comes off first in the oxidative pentose phosphate pathway.

The basis of the calculation is that it comes off equally from the two in the glycolytic pathway. Of course, there are many difficulties in this. The interpretation of our results is straightforward, based on the yields from glucose labelled in positions 1 and 6.

King: The limiting factor may not be the obvious one expected; for instance, the rates of the reactions in the glycolytic pathway may be quite different from those in the pentose phosphate cycle. If so, will it affect the calculation or interpretation?

Dickens: It is unlikely that in the glycolytic-citric acid pathway it would be at the same rate, because it goes through a whole chain of about 30 reactions before it is entirely liberated, and many of these may involve dilution of ^{14}C, whereas in the pentose pathway it comes off in the first step as $^{14}CO_2$ directly.

Short Communication:

RECENT OBSERVATIONS ON THE MECHANISM OF GLYCOGEN SYNTHESIS IN MUSCLE

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These experiments (Robbins, Traut and Lipmann, 1959) were started as an attempt to go back to the type of preparation which Krebs had used and where he found the maintenance effect of insulin on respiration, namely the pigeon breast muscle (Krebs and Eggleston, 1938). We wanted, however, to assay for glycogen synthesis and to look for an insulin effect on this reaction. We could not obtain any set-up that synthesized glycogen in a controllable manner with the originally used, coarsely cut muscle obtained with an instrument rather similar to the Latapie cutter. However, after some trial and error, a system was found using the conventional homogenization technique which appeared useful for our purpose. Although generally in the course of the experiment glycogen was partially degraded, the synthetic activity could be followed by way of radioactive glucose or glucose-6-phosphate incorporation into glycogen.

To get good incorporation, this system had to be supplied with muscle kochsaft which could be replaced by coenzyme A, while addition of pyridine nucleotides and adenosine triphosphate (ATP) had no effect. The effect of CoA and kochsaft appears, however, to be indirect since, as shown in Fig. 1, CoA addition maintained respiration on which the glycogen synthesis depended. Oxidative phosphorylation, which was the ultimate energy donor in this system, could not be replaced by ATP or an energy-rich phosphate feeder system. Perhaps the speed with which energy-rich phosphate has to be fed could only be obtained by the mitochondrial system. In any case, addition of dinitrophenol abolished incorporation. A further activator appeared to be aspartate, which maintains the level of adenosine phosphates; in its absence, partial degradation to inosinic acid was observed in the paper electropherogram.

Fig. 2 gives a more detailed view of the CoA effect. The reason for inhibition by higher CoA concentrations is not understood. It should be added that the mere addition of sulphydryl carriers such as glutathione or cysteine had no effect. The general metabolic

picture with and without CoA, which has some bearing on the theme of this symposium, is illustrated by Fig. 3, a radioautograph of an electropherogram. Samples were taken at the end of the experiment

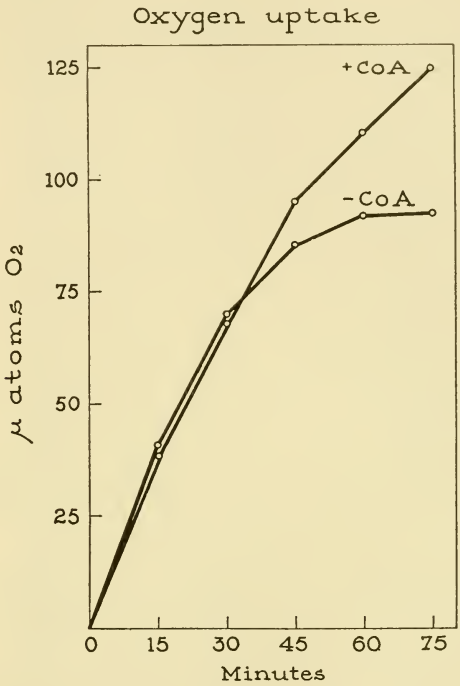

Fig. 1. Oxygen uptake by pigeon breast muscle preparations in the presence and absence of CoA. Experiments were carried out essentially as described by Robbins, Traut and Lipmann (1959). The Warburg flasks contained 1·2 ml. of a 1 : 4 homogenate of pigeon breast muscle. The final volume was 2 ml. and contained 4 μmoles of ^{14}C-glucose (2 μc) in addition to the homogenate. CoA was present at a concentration of 10 units/ml.

represented in Fig. 1. It may be seen on the radioautograph that without added CoA, glycogen is practically unmarked, while with maintenance of respiration by CoA it picks up a good deal of radioactivity. In contrast, the radioactive lactic acid, obviously formed from radioactive glucose, is weak in the presence of and rather

strongly marked in the absence of CoA, indicating a prevalence of glycolytic breakdown in the latter case. We were rather interested in the radioactive spot below the lactic acid which appears strongly in the CoA-containing sample, until it was identified as phosphoglycerol. The appearance of this compound under these conditions may be of interest but we did not wish to expand on this observation.

When we came to test insulin in this system, it proved to be

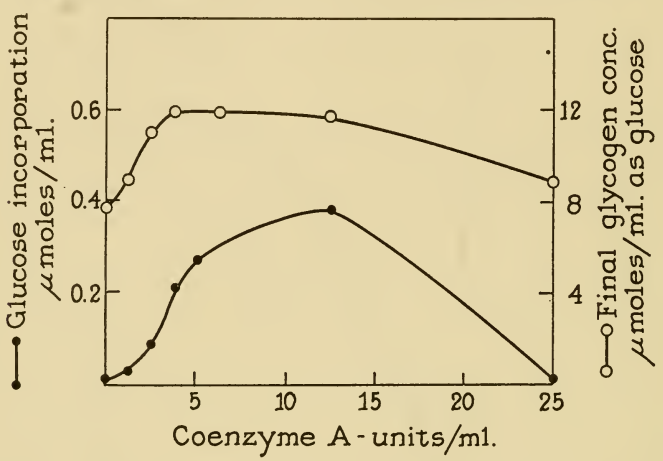

FIG. 2. Effect of CoA concentration on glucose incorporation into glycogen and on the maintenance of glycogen. Incubations were carried out for 60 minutes as described by Robbins, Traut and Lipmann (1959) and in Fig. 1. The reactions were stopped by adding an equal volume of 60 per cent KOH. Glycogen was isolated for counting and for chemical glycogen determinations essentially by the method described by Hassid and Abraham (1957).

completely inactive. Adrenaline, at a relatively high concentration, inhibits glucose incorporation into glycogen.

We come now to the results which have impressed us most, and which bear on the relationship betw en phosphorylase and glycogen synthesis. The experiment traced in Fig. 4 was carried out to explore the effect that an increase in phosphorylase *a* by addition of phosphorylase kinase might have on glycogen synthesis. It appears that an addition of kinase which maintains phosphorylase *a* at a higher level, almost completely abolishes glucose incorporation. In contrast, in the absence of kinase, the steady decrease in phosphorylase activity to practically zero coincides rather startlingly with an increase of glucose incorporation into glycogen. In Fig. 5,

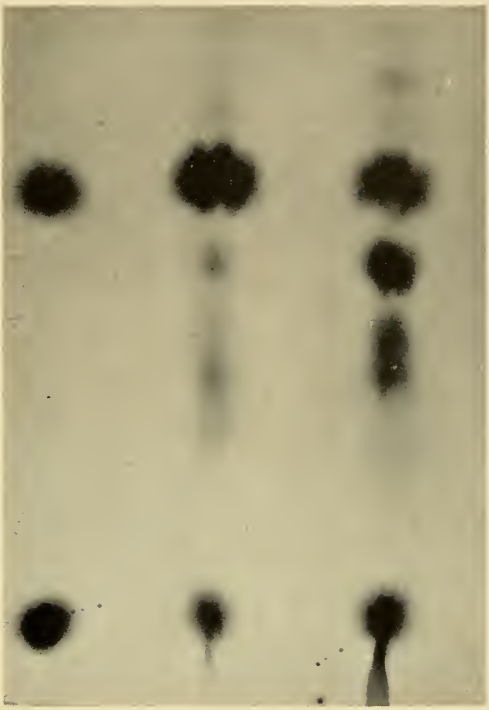

FIG. 3. Electrophoresis pattern of the products of glucose metabolism in the presence and absence of CoA. Aliquots were taken from the experimental flasks illustrated in Fig. 1 after 75 minutes and were placed in a boiling water bath for 2 minutes. After centrifugation, aliquots of the supernatant fluid were used for paper electrophoresis in 0·05 M citrate, pH 4·7.

[*facing page 190*

an analogous experiment is presented using glucose-6-phosphate as the glucose donor for incorporation. Although here some incorporation is maintained even with added kinase, its addition obviously depresses the incorporation. At the same time, as seen on the right-hand part of Fig. 5, it appears that with kinase added, glycogen

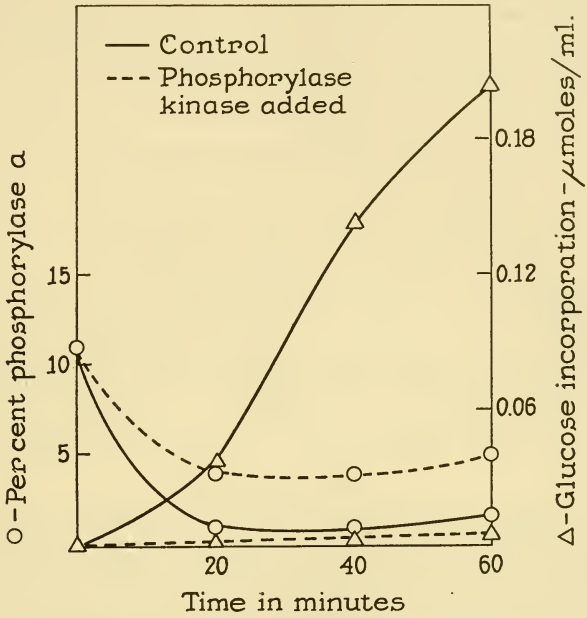

FIG. 4. Effect of phosphorylase kinase on the incorporation of glucose into glycogen. The experiments were carried out in Warburg flasks as described by Robbins, Traut and Lipmann (1959). The homogenates were supplemented with kochsaft (0·2 ml./ml.) and with crystalline phosphorylase *b* (Fischer and Krebs, 1958). Phosphorylase kinase was prepared according to Krebs and Fischer (1956). Incorporation was measured as described previously (Robbins, Traut and Lipmann, 1959) and phosphorylase was assayed according to Cori and co-workers (1955).

disappearance was accelerated. It may be noted that the induction period for incorporation with free glucose is absent with glucose phosphate. Apparently, conversion of glucose phosphate is more direct (cf. below).

The results of this experiment forced us to consider the possibility that phosphorylase *a* may not be concerned physiologically with

glycogen synthesis but rather with glycogen utilization. Such a proposition had also appeared to fit better with the results of Sutherland and his collaborators on adrenaline effects (Sutherland, 1956).

A very likely candidate for a system synthesizing glycogen more effectively than phosphorylase*, was the recently discovered uridine diphosphate glucose (UDPG)-linked glycogen synthesis of Leloir and Cardini (1957). Here, the equilibrium is much more in favour of synthesis than if glucose-1-phosphate is the glucose feeder (Cori,

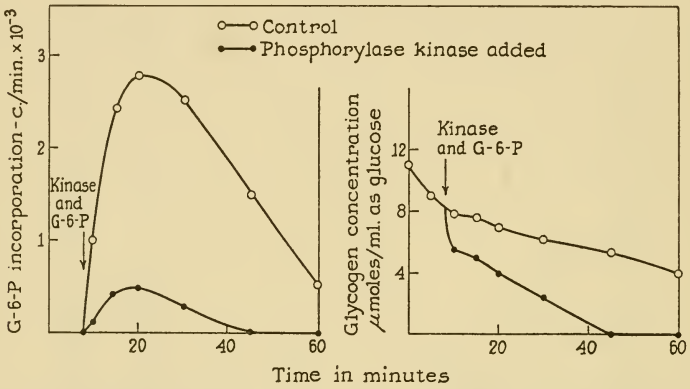

FIG. 5. Effect of phosphorylase kinase on incorporation of glucose-6-phosphate into glycogen and glycogen maintenance. The incubations were carried out in 125-ml. Erlenmeyer flasks. ^{14}C-glucose-6-phosphate and phosphorylase kinase were added after 8 minutes of incubation. Samples were withdrawn at the times indicated for incorporation measurements and chemical glycogen determination.

Cori and Green, 1943; Cardini, Leloir and Chiriboga, 1955). In the case of glycogen synthesis, the situation is even more favourable than in sucrose synthesis (Cardini, Leloir and Chiriboga, 1955) since the 1–4 glucosidic link in glycogen has a lower group potential than the 1–2 glucosidic link in sucrose. In partial confirmation of such a proposition, the synthesizing enzyme using the UDPG-glycogen pathway has been found in muscle in high concentration, and preliminary results also indicate the equilibrium to be almost completely on the side of glycogen synthesis† (Robbins, Traut and Lipmann, 1959).

* This paragraph includes data obtained during autumn 1958, in following up the results reported at the symposium.

† On a recent visit to the U.S.A., Dr. Leloir reported on studies on the muscle enzyme catalysing UDPG-linked glycogen synthesis (Leloir *et al.*, 1959).

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ALTERNATIVE PATHWAYS OF ELECTRON TRANSPORT

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Some years ago, the idea was put forward (Martius, 1954) that there may exist in the mitochondria two different routes for the transport of hydrogen atoms or electrons, respectively, between the pyridine nucleotides and cytochrome *c*, only one of them being linked with the enzymes which bring about phosphorylation. The so-called classical route, which leads from diphosphopyridine nucleotide (DPN) to cytochrome *c* reductase and then to cytochrome *c* or first to c_1, is not, in my opinion, connected with the formation of high-energy phosphate bonds. This should be the task of the second alternative pathway in which vitamin K plays an important rôle. These concepts, especially the one concerning the rôle of vitamin K, have not been accepted very enthusiastically by the experts, although Chance and Williams (1956), for instance, seem to agree on the concept regarding the possibility of the existence of two pathways for the hydrogen atoms. The main idea that, besides the known representatives of this class, vitamin K should react as an oxidation-reduction catalyst, was the one which seemed strange and unconvincing.

The present author's reasons for continuing to support this concept are given here, and its possible significance for the general problems of this symposium are discussed briefly. The crucial point of the scheme is the link between the pyridine nucleotides and vitamin K. Some time ago we reported (Martius and Strufe, 1954) the detection in mitochondria of an enzyme, phylloquinone reductase, which should represent the missing link. We now know that our first preparations con-

tained only small traces of this enzyme which for special reasons henceforth shall be called vitamin K reductase. In fact, we met with great difficulty in trying to enrich and prepare the substance in a pure state, and finally attained our goal only by combining several efficient methods which have recently been developed for the separation of proteins. Starting with extracts of beef liver, which is the best source of this enzyme, an overall purification of more than 4,000-fold had to be performed before an electrophoretically pure preparation was obtained. To our great surprise the new enzyme proved to be a hitherto unknown flavoprotein (Martius and Märki, 1957). The spectrum shows two peaks in the visible region, one at 380 mμ, the other at 456 mμ and a shoulder at 485 mμ. The normal peak is found in the u.v. region at 273 mμ. The prosthetic group of the enzyme proved to be flavin-adenine dinucleotide (FAD). The coenzyme can be reversibly split off by dialysis against distilled water. The activity can be fully restored by addition of a solution of authentic FAD. The identity of the coenzyme was further checked by means of the D-amino acid oxidase test for FAD, which was positive. There are remarkable differences in the enzymic behaviour of vitamin K reductase and cytochrome *c* reductase. Both can react in the reduced state with phylloquinone, but only the latter with cytochrome *c*. *p*-Chloromercuribenzoate (PCMBA) in a concentration of 10^{-4} M completely inhibits the Mahler enzyme whereas vitamin K reductase remains absolutely unaffected by the same inhibitor. Apparently the latter does not contain enzymically active SH-groups; on the other hand, it is inhibited by low concentrations of dicoumarol and other vitamin K antimetabolites. With 10^{-5} dicoumarol the inhibition rate is 100 per cent, with 10^{-6} M it is 95 per cent, and even when a 10^{-7} M concentration was used about 50 per cent inhibition was still observed. Thus, dicoumarol is a very potent inhibitor, the inhibition being of the competitive type. Substances which are related chemically or in other respects, e.g. dinitrophenol (DNP), antimycin, or hydroxynaphthoquinone derivatives like SN5949, are, however, without any

effect. This is an important fact, for it demonstrates that in the case of vitamin K we are really dealing with the physiological substrate of this enzyme.

Another important property of vitamin K reductase is its high activity. In low concentrations and with phylloquinone as acceptor this enzyme shows a turnover number of about 1,200,000 per molecule per minute. This calculation is based on the flavine content. The molecular weight is not yet known with certainty. The sedimentation coefficient (S_{20}) is very low, about 3·1. Unfortunately we have not yet been able to estimate the diffusion coefficient. Assuming that the molecule has a globular shape, the molecular weight would be in the region of 30,000.

Though values for the content of cytochrome *c* reductase are known only for pig heart, the concentration in the tissues of both enzymes seems to be of the same order of magnitude. This fact, together with the above-mentioned high turnover number of vitamin K reductase, points to the important rôle of this enzyme in cell respiration. A further peculiarity of vitamin K reductase is the fact that it is not only reduced by reduced diphosphopyridine nucleotide (DPNH), but also by reduced triphosphopyridine nucleotide (TPNH) (Martius and Märki, 1957), which implies that TPNH could also contribute to the formation of high-energy phosphate bonds to the same extent that DPNH does. This would not be in agreement with the recent statements by Vignais and co-workers (Vignais and Vignais, 1957; Vignais, Vignais and Bartley, 1957). However, the whole problem remains open and should be carefully reinvestigated.

We have recently carried out a simple experiment which is really a repetition, with better facilities, of an older experiment of ours. Using liver mitochondria from highly vitamin K-deficient chickens we have compared the P/O ratios which one obtains with β-hydroxybutyrate and with succinate as hydrogen donors (Table I). In the latter case quite normal values are obtained, which seem to indicate that for steps (2) and (3) of the phosphorylation sequence the pathway for the

electrons and the system of the phosphorylation enzymes involved are intact and unaffected by the vitamin deficiency. But with β-hydroxybutyrate as substrate the P/O ratios are lower than in normal mitochondria, and even lower than with succinate and the same mitochondria. This disturbance, which *in vitro* can be overcome by the addition of vitamin K_1, can only be explained by assuming that there is a gap in the normal phosphorylating pathway; the gap must be between the pyridine nucleotides and cytochrome *b*, for from there, as the experiments with succinate show, the electron transport

Table I

Oxidative phosphorylation in vitamin K-free chicken liver

	μatom oxygen	*μatom phosphorus*	*P/O*
β-Hydroxybutyrate	2·52	3·53	1·40
Succinate	6·32	12·17	1·93
β-Hydroxybutyrate	2·95	3·04	1·02
Succinate	5·92	9·86	1·66
β-Hydroxybutyrate	2·52	3·33	1·33
Succinate	5·29	9·39	1·77
β-Hydroxybutyrate	2·00	2·85	1·42
Succinate	6·35	12·28	1·93

is quite normally linked with phosphorylation. Obviously the hydrogen atoms or electrons coming from the reduced pyridine nucleotides must circumvent this gap, i.e. they must make their way via the cytochrome *c* reductase. Entering the phosphorylating pathway at the cytochrome *c* level, they can bring about only one single phosphorylation. Similar conclusions have recently been drawn from experiments with irradiated mitochondria (Beyer, 1958), and in bacterial systems, also, vitamin K is necessary for electron transport and phosphorylation, as has been shown by Brodie, Weber and Gray (1957).

Thus, it appears that the scheme postulated in 1954 is now well enough established and we may consider what conclusions can be drawn from it. First of all, if two different pathways,

a phosphorylating one and a non-phosphorylating one, are provided in the cell, this fact must have physiological significance. Apparently, the cell is prepared for the oxidation of hydrogen without coupled phosphorylation, at least in the first two steps of the respiratory chain. The problem now arises of how the electrons or hydrogen atoms are directed in one or the other direction. In normal, intact mitochondria, they seem to take exclusively the pathway which is coupled

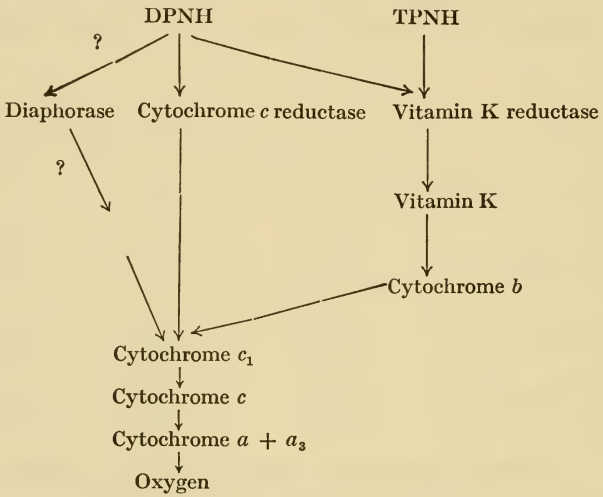

FIG. 1.

with phosphorylation and leads via vitamin K reductase, vitamin K and cytochrome *b* (Fig. 1). This pathway, however, appears to be very sensitive to any alteration of the internal structure of the mitochondria, probably because some of its constituents are structurally firmly bound. If their combination is disturbed, the electrons, as in the case of the gap produced by vitamin K deficiency, have to take the emergency exit, as it were, via cytochrome *c* reductase to cytochrome *c*. The same seems to hold true if, instead of internal formation of DPNH, DPNH is added from outside to the mitochondria. In this case, as Cooper and Lehninger (1956*a*) have demon-

strated with mitochondrial fragments, only a very low P/O ratio is obtained. They found 0·24 in one case instead of 1·7 which they had obtained when the DPNH was formed internally by the action of β-hydroxybutyrate dehydrogenase. In this respect, their results are in good agreement with the concept held by the present author. This, however, is not the case concerning the mode of action of dicoumarol. It has already been suggested (Martius, 1954) that the uncoupling action of this compound may be explained by assuming that the phosphorylating pathway is blocked between vitamin K and its reductase. This must be the case, because vitamin K reductase, as shown above, is completely blocked by 10^{-6} to 10^{-5} M dicoumarol. On the other hand, the fact that the phosphorylation step between cytochrome *c* and oxygen is likewise affected by dicoumarol (Cooper and Lehninger, 1956*b*) cannot be explained in this way. That question remains open.

In conclusion, if alterations of the internal structure of the mitochondria result in shifting of the flow of electrons from one pathway to the other, this could also be the reason for the uncoupling action of thyroxine. The well known fact that thyroxine causes a swelling of the mitochondria shows that it has an influence on the structure. Hitherto, it was assumed that this action was directed only to the structure or the function of the membrane; but recently it has been shown by Park, Meriwether and Park (1958) that, contrary to the earlier observations of Lehninger, mitochondrial fragments are also affected by the hormone. If this finding were confirmed, then it could mean that the internal structure of the mitochondria might also be disturbed by the hormones, and the uncoupling effect of thyroxine could be explained in this way.

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DISCUSSION

Magasanik: In intact mitochondria the flow of electrons is controlled by vitamin K. What prevents the other pathway from functioning? You have not postulated any particular inhibitors; is there any evidence that something is produced in the vitamin K pathway that would tend to inhibit the cytochrome *c* reductase pathway?

Martius: I do not know.

Potter: Is there any available information about the Michaelis constants, for instance, for DPNH and cytochrome *c* reductase versus vitamin K reductase?

Martius: The constants have not yet been determined as far as vitamin K reductase is concerned.

Lehninger: The uncoupling in the two major spans of the chain is interesting since both have the same sensitivity to dicoumarol as your purified enzyme. In other words, 10^{-6} M produces virtually complete uncoupling at all three levels in the chain. It is possible that there are two actions of the compound. An alternative explanation may be that vitamin K may not be in the respiratory chain *per se*, but may be "parasitic" at three different points.

Siekevitz: Another explanation could be that there are quinones besides the vitamin K, which come in at the cytochrome *c* level, or even at the succinic dehydrogenase level.

Krebs: If it occurs at three levels, as you suggest, would it mean that these three substances must have different oxidation-reduction potentials appropriate to the level at which they were operative? I suppose that would be necessary and it would also be feasible.

Lehninger: It is a different way of looking at the mechanism of uncoupling and phosphorylation. It is remarkable that different vitamin K derivatives have different O–R potentials. It might conceivably fit in at three different levels.

Lipmann: I have wondered about these three levels and the all-or-none effect of the uncouplers. I think Dr. Slater's proposition made on X∼I interchange may be the answer. As soon as a transformation of O–R potential into another form of energy has occurred, then the exchange can occur across the O–R potentials. The phosphate can thus exchange, and it does not matter at what potential level. We have to consider that very seriously.

Siekevitz: It could also be that in all the phosphorylation steps everything will funnel into one X compound, and the all-or-none effect could be accounted for in this manner.

Lipmann: Prof. Lehninger has shown that there are different ways of uncoupling, and they all seem to be all-or-none. Then one cannot think of it funnelling into one step but rather into two steps.

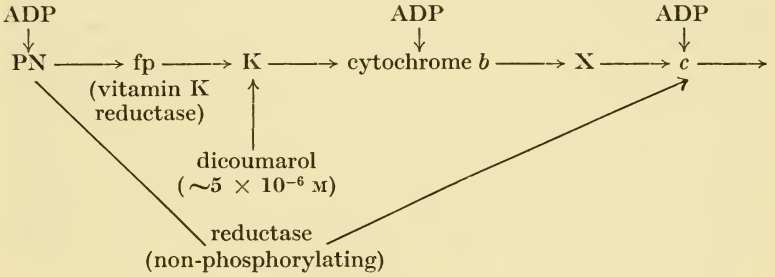
Estabrook: If the vitamin K reductase is not inhibited by *p*-chloromercuribenzoate, do you get oxygen consumption in the presence of *p*-chloromercuribenzoate during phosphorylation? If the by-path is blocked, then all the electrons would be funnelled to the vitamin K reductase, using DPNH as substrate.

Martius: As yet we have not done this experiment. There are many enzymes which could be blocked by *p*-chloromercuribenzoate.

Lipmann: Does DNP inhibit the reductase?

Martius: No, it does not.

Chance: I was interested in the system which Prof. Martius has discussed and which we represent as follows:



We have an opportunity to test more critically the hypothesis presented and to see whether the site of dicoumarol action would be specific at the point indicated. While both dicoumarol and ADP separately accelerate the flow of electrons through this system, Prof. Martius' hypothesis would require that a block with dicoumarol would activate the non-phosphorylating reductase pathway. We compared the effect of dicoumarol and ADP on phosphorylating liver mitochondria. If dicoumarol blocks at the point indicated, it would cause a diminution of the flow of electrons to cytochrome *b*. The steady state oxidation-reduction level of cytochrome *b* would then go considerably more oxidized, but that of cytochrome *c* would stay about the same. In the presence of dicoumarol (5×10^{-6} M) the bands of both cytochromes *b* and *c* show approximately the same changes (Fig. 14, p. 109). Furthermore, the electron flow was not blocked between "fp" and "b". It appears that dicoumarol has no selective action on the possible site of the "K reductase" or K itself. Thus, the proposed sequence is not supported by this particular experimental test.

Potter: I wonder whether, at a dicoumarol concentration as low as

5×10^{-6} M, the percentage inhibition is related to the amount of vitamin K reductase used. In other words, is it practically irreversible?

Martius: It is a competitive inhibition.

Potter: So it is not likely to be related to the amount of the enzyme.

How does one explain the low yield of phosphate-bound energy with TPNH as compared with DPNH? From your Fig. 1 it appears that it would be much easier to get a low yield with DPN than with TPN. Could vitamin K reductase act as a transhydrogenase?

Martius: The transhydrogenase activity of vitamin K reductase is under investigation.

Lehninger: When I commented on the dicoumarol uncoupling at three different levels, I suggested the possibility that the vitamin K-containing substance may be "parasitic" along the chain at three different points. You have said that this is not possible. Have you some experimental reason for excluding a "parasitic" vitamin K-containing carrier at each of the three levels? Supposing we had, at each of the three coupling sites, the possibility that these vitamin-K-containing "parasitic" molecules, which can change their oxidation-reduction state, were in some kind of equilibrium with the real carriers, and that the O–R potentials of the three different vitamin K derivatives might fit those of the carriers at the coupling sites; in other words, a kind of condenser system. This could account for dicoumarol uncoupling at three different sites. Recently Todd and his co-workers have speculated that quinones might be involved in phosphorylation (Clark, V. M., Kirby, G. W., and Todd, Sir A. (1958). *Nature, Lond.*, **181**, 1650). I am just putting their suggestion together with the possibility of vitamin K acting at three different sites. There may be some kind of sympathetic "parasitism" between the O–R states of these three different vitamin K compounds at three different sites here, which may be connected mechanistically with phosphorylation.

Martius: I do not believe that vitamin K acts in three different steps, but only in the first step, because its reduction potential is between cytochrome *b* and the flavoproteins. The naphthoquinone group is the functional group of vitamin K. It must be this group which is oxidized and reduced.

Potter: In 1940 I proposed a definition of a hydrogen transport mechanism, namely, that if it is a hydrogen transport mechanism it has one system which reduces it, and a different system which oxidizes it (1940, *Medicine*, **19**, 441). The difference in your parasitic system, which is analogous to a condenser, depends on whether there is only one system that can oxidize it and reduce it. The crucial experiment for the argument is whether or not the reduced vitamin K is readily oxidized by cytochrome *b*.

Martius: It is, and very rapidly.

Potter: If it is, then you have fulfilled the requirements for hydrogen transport, and it is not a dead-end street, which I gather is Prof. Lehninger's definition of a parasite.

Lehninger: The condenser analogy was unfortunate. I meant these things to be in equilibrium, not necessarily in series, but in parallel;

but separated from each other; and also three different kinds of vitamin K derivatives with three different potentials. This scheme, of course, is all manufactured out of whole cloth, right here on the spot, and I don't really believe it. I just want to account for the fact that dicoumarol uncouples at all three phosphorylation sites.

It seems to me that there is a very important discrepancy between this fact and Prof. Martius' views.

Krebs: Prof. Martius (1956, *In* Proc III int. Congr. Biochem., p. 1. New York: Academic Press) left out the flavoprotein in his earlier schemes, when he introduced vitamin K. Is he ready to put it back again, having discovered that the vitamin K reductase is a flavoprotein? There ought to be room for both in the series.

Martius: Four years ago, we believed that vitamin K reductase was not a flavoprotein, but now we know that it is a flavoprotein. Now we have at least two flavoproteins in the respiratory chain.

Krebs: It has always been understood that flavoprotein is a generic name; there may be several which may react in series.

Slater: Prof. Potter's point as to whether reduced vitamin K_1 was oxidized again fast enough to account for it being in the main chain interested us very much. We tried to investigate that but could only use reduced vitamin K_3 as a model, and that was very rapidly oxidized, getting right into the respiratory chain. We could not use reduced vitamin K_1, because we did not know how to make it at the time. Does your answer to Prof. Potter's question mean that reduced vitamin K_1 can be oxidized by oxygen with a mitochondrial preparation?

Martius: The answer is the same as to Prof. Potter's question. We were able to demonstrate the rapid oxidation of reduced vitamin K_1 by cytochrome *b* (cytochrome *b* preparation of Stotz) with exclusion of oxygen.

Slater: What is the final hydrogen acceptor—oxygen or cytochrome *b*?

Martius: Cytochrome *b*.

Lipmann: I was always rather impressed by the naphthoquinone possibility. The problem of a converter mechanism of electron potential and phosphate potential was raised quite a while ago (Lipmann, F. (1946). *In* Currents of Biochemical Research, p. 137. Ed. Green, D. E. New York: Interscience Publishers). If we want to bring some reason into the oxidative phosphorylation, we have to keep in mind that we need such converter mechanisms. We need the mechanism which converts electron potential into phosphate bond potential. I still hope that vitamin K will somehow fit in. I cannot quite see how it can fit only into one place: perhaps quinones are on several places and perhaps dicoumarol would inhibit them all.

King: If reduced K_1 can be oxidized by cytochrome *c*, why do you put *b* and *x* into the chain, especially if the system is not antimycin-sensitive? Secondly, is your cytochrome *c* reductase the same as Mahler's?

Martius: It is true that reduced vitamin K_1 can react with cytochrome *c*. In that case, however, we could expect a maximal P/O ratio in mitochondria of only 2. Our vitamin K reductase is not identical with Mahler's cytochrome *c* reductase.

Siekevitz: Prof. Lehninger, did you have in mind with the parasitic relationship that perhaps K_1 does not act as an O–R compound, but that it may be one of the X's?

Lehninger: Prof. Potter's statement about the requirements of what constitutes a chain does not necessarily exclude the enzyme described by Prof. Martius as such a "parasite". Perhaps it is the oxidation/reduction state of the naphthoquinone, as Prof. Lipmann has just mentioned, that conditions the effectiveness of X, Y and Z to participate in phosphorylation. We have presented evidence elsewhere that the oxidation/reduction state of the respiratory chain and/or other things associated with that chain, condition the rate of the ^{32}P exchange and ATPase activity (Wadkins, C. L., and Lehninger, A. L. (1957). *J. Amer. chem. Soc.*, **79**, 1010; 1959, *J. biol. Chem.*, in press). I do not think that your point excludes such a parasitic relationship. Prof. Martius' enzyme may be one of these that happens to fit at the level of cytochrome *b* or *c*. There may also be some other enzymes which happen to fit further along the chain—that is pure speculation.

Slater: We are toying with the idea that vitamin E is one of the X's or the I's. I do not see where you fit in the phylloquinone reductase in this scheme if you make vitamin K an X or an I.

Siekevitz: Could it not be that vitamin K_1 acts as an X only; and whether it is oxidized or reduced is determined by its reductase and thus the reductase would determine its effectiveness in phosphorylation?

Slater: This discussion turns now on the experimental results: whether the state of oxidation-reduction in the respiratory chain does or does not have an influence on the reaction.

LIMITING FACTORS IN GLYCOLYSIS OF ASCITES TUMOUR CELLS AND THE PASTEUR EFFECT*

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Before we can investigate regulatory mechanisms of metabolic pathways and explore how they are influenced by hormones, drugs or poisons, we should have a detailed knowledge of the metabolic process itself. Let us start by expressing doubts as to whether we have the basic knowledge of intracellular metabolism needed for such studies. On the other hand, we must admit that considerable information regarding regulatory mechanisms has already been obtained, as is apparent from the discussions during this symposium. Perhaps we are somewhat in the position of the talented student of the Talmud, who was known for his brilliant answers. One day he went to his equally talented friend, who was known for his brilliant questions, and requested: "Please ask me a good question, I have a wonderful answer."

During the past three years some efforts have been made in our laboratory at reconstructing life, as Dr. P. P. Cohen jokingly calls it. We have taken individual, highly purified enzymes of glycolysis and have put them together with mitochondria in order to study their interaction. We share Dr. Cohen's scepticism regarding the resemblance of these reconstructed systems to intracellular metabolism. Since we have recently reviewed some of these efforts of our reconstruction period (Racker and Gatt, 1959), we need not discuss them

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here in detail. It may suffice to say that we have simulated phenomena such as the Pasteur and Crabtree effects in artificial mixtures of respiring mitochondria and glycolytic enzymes. We have reconstructed a pentose phosphate cycle which oxidizes all six carbons of glucose-6-phosphate (Couri and Racker, 1958, unpublished) and it has been possible to demonstrate formation of fructose-1 : 6-diphosphate from pyruvate, thus reversing a segment of glycolysis (Krimsky, 1959). But we would emphasize that we regard these reconstructed systems as models only, from which we can learn, by variation of their components, how these phenomena might be brought about. In order to gain information as to how these processes are actually accomplished within the cell, we must turn to the cell itself.

We have chosen for study the ascites tumour cells (Ehrlich, tetraploid) because they exhibit both Pasteur and Crabtree effects and because they are very suitable for metabolic studies. Although various ascites tumours have been extensively studied, we have found little information regarding the limiting factors of their carbohydrate metabolism.

We proceeded, therefore, to explore systematically their enzymes, coenzymes and metabolites. The profile of the glycolytic enzymes in extracts obtained by centrifugation at 100,000 ***g*** for 30 minutes, and in mitochondrial particles centrifuged down at 10,000 ***g*** was determined by measuring enzyme activities under standard conditions at pH 7·4 in the presence of excess substrate and coenzymes. The experimental data, which are being published elsewhere, can be summarized as follows. The bulk of the glycolytic enzymes was found in the high-speed supernatant solution (extract). Only hexokinase was found to be concentrated in the particles (up to 60 per cent of the total) and to have a specific activity four- to nine-fold that of the extract. Small amounts of phosphofructokinase and aldolase activity were also associated with the particles; the activities of the other glycolytic enzymes could be accounted for by the amount of trapped supernatant fluid in the washed mitochondria. The profile

of enzyme activities in the extract showed some features of interest. Hexokinase and phosphofructokinase, the adenosine triphosphate (ATP)-utilizing enzymes of glycolysis, had the lowest capacity. 3-Phosphoglycerate (PGA) kinase and pyruvate kinase, the ATP-forming enzymes, as well as lactic dehydrogenase, had the highest capacities, between 10 and 40 times that of ATP-utilizing enzymes. It should be emphasized that the capacity of an enzyme, defined as its activity, measured in the presence of excess substrate and coenzymes, is not representative of its rôle in overall glycolysis, but conveys information with respect to its potentialities in competitive systems. For instance, it was found that a Pasteur-like effect can be produced by the addition of excess mitochondria to a reconstructed system of glycolysis. When the distribution of glycolytic enzymes was similar to that found in extracts of ascites cells, namely, with PGA kinase and pyruvate kinase present in high concentrations, a pronounced inhibition of respiration (Crabtree-like effect) could be obtained. With limiting amounts of these adenosine diphosphate (ADP) transphosphorylating enzymes, no Crabtree-like effect was observed although glycolysis was proceeding at an appreciable rate. This can be readily understood since the experiments were so designed that the regeneration of ADP from ATP was the rate-limiting step for both respiration and glycolysis. In order to compete with the efficient phosphorylation of ADP due to oxidative phosphorylation, relatively large amounts of transphosphorylating enzymes were required in the reconstructed system.

The next step was to measure overall glycolysis in crude extracts and homogenates of ascites cells. They were fortified with cofactors and inorganic phosphate in order to study the rate-limiting enzyme. It was found that, depending on experimental conditions, there could be 2 or 3 enzymes limiting the rate of glycolysis. The limiting factors were determined by adding individual, highly purified enzymes of glycolysis to the crude extract. Their effect on lactic acid production is shown in Table I. It can be seen that in dilute extracts (1 mg.

protein/ml.) phosphofructokinase and glyceraldehyde-3-phosphate (G-3-p) dehydrogenase were the major limiting enzymes of glycolysis while hexokinase was the major pacemaker in concentrated extracts. Addition of other glycolytic enzymes had no effect.

Table I

Rate-limiting factors in tumour extracts

Experiments were carried out in a final volume of 0·6 ml. containing 0·6 mg. or 2·4 mg. extract protein (12,000 *g* supernatant solution); 10 μmoles Tris buffer, pH 7·4; 4 μmoles $MgCl_2$; 10 μmoles potassium phosphate buffer, pH 7·4; 1 μmole DPN; 2 μmoles ATP; 6 μmoles KCl and 8 μmoles glucose. Incubated at 30°C for 20 minutes with shaking.

	μmoles Lactate	
Additions	0·6 *mg. protein*	2·4 *mg. protein*
—	0·31	2·5
Hexokinase	0·30	3·8
Phosphofructokinase	0·62	3·4
Aldolase	0·34	2·7
G-3-p dehydrogenase	0·63	3·1

If we compare the glycolytic rate of these fortified extracts with the glycolysis of intact cells (Table II) it becomes apparent that there is a large excess of glycolytic potential in the ascites cells. We can therefore draw our first conclusion, or

Table II

Comparison of glycolytic rates of extracts and intact tumour cells

With extracts the experimental conditions were similar to those in Table I with 2·4 mg. protein per 0·6 ml. With intact cells (10 mg. protein) the reaction mixture contained in a final volume of 0·6 ml: 20 μmoles Tris buffer, pH 7·4; 50 μmoles NaCl; 12 μmoles KCl; 3 μmoles potassium phosphate buffer, pH 7·4; and 6 μmoles glucose. Incubated at 30° for 25 minutes. Results are expressed as μmoles per 140 mg. cell protein (or that amount of extract obtained from 140 mg. cell protein) per hour.

Preparation	*Gas phase*	*Lactate production*
Intact cells	Air	68
	N_2	120
Extract	Air or N_2	360

should we say exclusion: the rate of either aerobic or anaerobic glycolysis in intact ascites tumour cells is not limited by the capacities of the glycolytic enzymes.

Let us turn now to what we call studies of intact cells. We have tried to investigate cellular metabolism by allowing ascites cells to utilize glucose under a variety of conditions. After short time intervals, the metabolic process was interrupted, e.g., with perchloric acid, and the concentrations of adenine nucleotides, intermediates, phosphate, etc., were determined in the extract. We realize fully that this experimental approach is not an ideal one, but unfortunately it is the only one available at present for studying most of the intracellular components. Let us remain cognizant of the fact that by turning from studies with extracts to "intact cells", we have but delayed the process of producing artifacts which we believe are inseparably attached to any procedure involving the disruption of the cell structure. But at least we produce by this approach an artifact of another kind, and the knowledge we obtain bears perhaps a closer relationship to cellular metabolism than that derived from studies with extracts. We can but hope that by collecting data from a large variety of artifacts we shall be able to extract the information necessary to obtain a composite picture of intracellular metabolism. Table III will serve to illustrate what information we attempt to obtain from such an approach.

Table III

RATE OF GLYCOLYSIS AND INTRACELLULAR PHOSPHATE AND ADP LEVELS

Incubation was made in Warburg vessels at 30°C for 40 minutes. Results are expressed as μmoles/140 mg. protein.

			Intracellular conc.	
Additions	*Gas phase*	*Lactate*	*Pi*	*ADP*
Glucose	Air	47	3·8	0·81
Glucose	N_2	78	5·5	1·00
Glucose + DNP*	Air	102	6·9	1·06

* Final concentration = 1×10^{-4} M.

Anaerobic conditions or the presence of 2 : 4-dinitrophenol (DNP) resulted in a marked stimulation of lactic acid production in ascites cells. Determination of the intracellular content of phosphate and adenine nucleotides revealed that pronounced fluctuation in inorganic phosphate (Pi) levels occurred which were roughly proportional to the rate of glycolysis. Similar changes in ADP levels were observed but they were neither as striking nor as consistent as the Pi levels. To explore the possibility of phosphate as a limiting factor of glycolysis, the extracellular Pi concentration was raised, as shown in Table IV. There was a marked stimulation of lactic acid production when the Pi level in the suspension medium was increased. Inclusion of adenosine monophosphate (AMP) in the medium had no effect on glycolysis, although increments of intracellular adenine nucleotides were noted under these conditions.

Table IV

Effect of extracellular Pi concentration on glycolytic rate

Cells were incubated in air with glucose at 30°C for 30 minutes. Results are expressed as μmoles/140 mg. protein.

μmoles Pi per ml. of medium	*Lactate production*	*Intracellular Pi conc.*
4	50	4·6
40	104	7·7
80	130	9·9

In view of these findings it was decided to investigate the rate of phosphate entrance into the ascites cells. This was done by measuring both intracellular Pi concentrations as well as ^{32}P distribution under various experimental conditions. As illustrated in Table V, the transport of Pi into the cells is dependent on a functional energy-yielding process such as respiration or glycolysis. Respiration becomes ineffective for Pi transport in the presence of DNP, a known uncoupler of oxidative phosphorylation. Glycolysis-linked transport is abolished on addition of iodoacetate.

We arrived at the second conclusion: in ascites cells the

Table V

^{32}P UPTAKE BY ASCITES TUMOUR CELLS

Cells (30 mg. protein) were incubated with ^{32}P—Pi (20,000 cpm/μmoles) at 30°C for 30 minutes.

Addition	*Gas phase*	*cpm entered (with 5 μM-Pi/ml.)*	*cpm entered (with 40 μM-Pi/ml.)*
—	N_2	5,900	34,000
Glucose	N_2	23,300	90,000
—	Air	18,000	60,000
Glucose	Air	33,000	105,000

intracellular concentration of Pi is a major limiting factor of glycolysis. The entrance of Pi into the cell appears to depend on an energy-yielding process and is sluggish compared to the rate of glycolysis.

In consequence of the above, the addition of glucose to

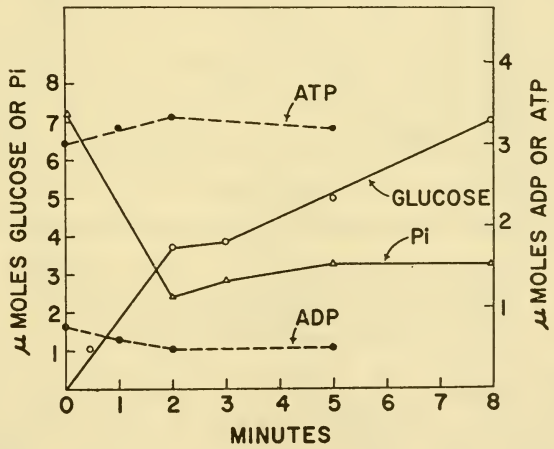

FIG. 1. Rate of glucose uptake and changes in intracellular concentrations of ATP, ADP and Pi
Glucose (3·7 μmoles/ml.) was added after 5 minutes of preincubation at 30°. Results are expressed as μmoles/ml. of packed cells.

ascites cells results in a continuous lowering of the intracellular Pi level until a new steady state is reached as shown in Fig. 1. As expected from the high Pi concentration, the initial uptake

of glucose is very rapid. Then follows a short period of almost complete cessation of uptake, a phenomenon first observed by Chance and Hess (1956). The resumption of glucose uptake proceeds then at a rate considerably below the initial one. The complete block of glucose uptake, despite a high intracellular concentration of ATP, was a rather puzzling feature

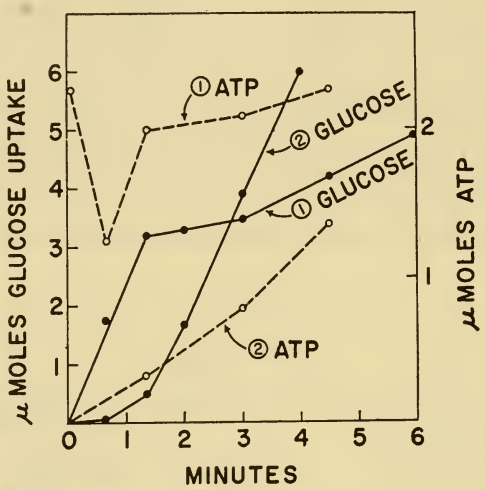

Fig. 2. Effect of DNP on the kinetics of glucose uptake
Experimental conditions were as in Fig. 1, except that the ascites cells were preincubated for 15 minutes with or without DNP (10^{-4} M). Curve 1 without, curve 2 with DNP.

of this phenomenon. In order to explore this further, the cells were pretreated with DNP to allow the intracellular ATP to be depleted. After addition of glucose, the rate of glycolysis and the levels of ATP were determined at frequent intervals (Fig. 2). After a short lag, the rate of glucose uptake was maximum at a time when the ATP levels were one-fourth that of the DNP-less control, which glycolysed at a considerably slower rate.

It seems apparent that the level of ATP, as measured in the extract after breaking up the cells, cannot be correlated to its

functional availability for the hexokinase reaction. It is possible that the impairment of glucose utilization is due to the aerobic accumulation of an inhibitor which, however, is not formed in the presence of DNP. Glucose-6-phosphate actually exerts a pronounced product inhibition on hexokinase obtained from ascites cells and, interestingly enough, the intracellular concentration of this compound is greater aerobically than anaerobically, in particular shortly after the addition of glucose. However, at no time is the intracellular concentration of glucose-6-phosphate sufficiently high to account for the inhibition of glucose uptake. Moreover, glucose-6-phosphate could not affect lactate production unless it inhibits, directly or indirectly, a step subsequent to its formation. It is deemed more likely that the inhibition of glucose uptake is due to a structural non-availability of ATP. Whatever the detailed mechanism of this inhibition phenomenon, we can come to our third conclusion: glycolytically produced ATP is more efficiently utilized for glucose phosphorylation than ATP produced during oxidative phosphorylation.

In Table VI the effect of high Pi concentrations on the Pasteur effect is shown. It can be seen that high concentrations of extracellular Pi diminish the magnitude of the Pasteur effect, both in respect to lactic acid production and glucose uptake.

Table VI

THE EFFECTS OF HIGH Pi CONCENTRATIONS ON THE PASTEUR EFFECT

Incubation made at 30°C for 30 minutes. The results are expressed as μmoles/140 mg. protein/hour.

μmoles Pi per ml. medium	*Gas phase*	*Lactate*	*Percentage Pasteur effect*	*Glucose*	*Percentage Pasteur effect*
3	Air	80	58	72	47
3	N_2	190		135	
30	Air	161	40	123	29
30	N_2	270		174	
60	Air	230	27	160	19
60	N_2	314		197	

We come to conclusion four: the Pasteur effect in ascites cells can be released to a considerable extent by high extracellular phosphate. The Pasteur effect may be visualized to be due to the low availability of intracellular Pi under aerobic conditions which limits glycolytic synthesis of ATP and therefore limits efficient glucose uptake.

The total intracellular concentration of Pi represents a balance of the processes which esterify Pi, on the one hand, and release it from phosphate esters or transport Pi from the outside, on the other hand. However, it has become apparent from our studies that the total Pi, as well as the ATP concentration, does not seem fully available for glycolysis, thus indicating a compartmentation of the cell. We shall come back to this point shortly. Here, mention should be made of the work of other investigators who have carried out similar work with yeast or tumour cells and who have come to similar conclusions. First of all, we should give credit to the brilliant vision of Johnson (1941) and Lynen (1941) who first proposed that Pi is a key compound in the Pasteur effect. Lynen and Koenigsberger (1951) have pursued this problem very actively and have postulated aerobic inavailability of ATP for hexokinase. Reference should also be made to the recent work of Kvamme (1958) since his studies have in some respects paralleled ours.

Having dealt with some specific questions which were susceptible to experimental approach, we would like to turn now to some features of cellular metabolism which are more difficult to investigate. Ascites cells, in common with other cells, can glycolyse and respire in a phosphate-free medium. The equations for these metabolic processes are unbalanced unless we include the breakdown of ATP to ADP and Pi. Moreover, in order to maintain a steady state this process of ATP breakdown must proceed at a rate equal to its synthesis. All enzyme systems which catalyse the breakdown of ATP to ADP and Pi can be referred to as ATPase in its broadest sense. This includes the biosynthesis of proteins, carbohydrates, etc., transport mechanisms and any other work

which utilizes either ATP itself or a compound which requires ATP for synthesis. It includes also the enzymes which cleave ATP to AMP and pyrophosphate, since pyrophosphatase and adenylic kinase (*myo*-kinase) which generate Pi and ADP from pyrophosphate and AMP are universal cellular components. It includes also the enzymes which simply hydrolyse ATP to ADP and Pi, although some doubts have been expressed as to whether these "wasteful enzymes" function intracellularly in this manner.

It has been mentioned before and emphasized by Lynen and Koenigsberger (1951) that in a steady state the rates of cellular phosphorylation and dephosphorylation must be equal. Since we have found it quite difficult in our reconstructed systems to balance accurately the phosphorylating enzymes by the addition of ATPase, our already boundless admiration of Nature's accomplishments has received a further stimulus. How do cells maintain the oxidative and glycolytic rates under such a variety of conditions and adjust ATPase activity to follow suit? We have no real answer to this question, but feel vaguely that the fashionable feedback mechanisms play a significant rôle. We have some clues from our cell-free system as to how ATPase activities might be adjusted, but no clues as to whether these mechanisms are operative intracellularly. We have observed that a mitochondrial ATPase, which is minimal when assayed by conventional procedures, can be made to manifest itself by the continuous removal of ADP. This can be accomplished by an ATP-regenerating system consisting of phospho*enol*pyruvate and pyruvate kinase. ADP, which is known to inhibit ATPase activity (Kielley and Kielley, 1953) can be thus visualized to regulate the breakdown of ATP. If such a mechanism operates in respiring mitochondria, this ATPase activity should increase during oxidative phosphorylation. It is not relevant for this concept whether mitochondrial ATPase catalyses a truly hydrolytic reaction or is a metabolic expression of some work that has been accomplished, e.g. transport of potassium. Even if the mitochondrial ATPase activity

were simply hydrolytic and apparently wasteful, it could represent a most vital mechanism by allowing continuous operation of the Krebs cycle, though at a reduced rate. Perhaps it is superfluous to remind this group that the Chairman of this symposium has frequently emphasized that the tricarboxylic acid cycle has biosynthetic functions (glutamic acid, porphyrins, etc.) in addition to its rôle in oxidation.

The second example concerns an ATPase activity which we have encountered in the presence of highly purified glycolytic enzymes. Dr. Krimsky made the observation that both transphosphorylating enzymes of glycolysis can be readily reversed provided the ATP/ADP ratio is maintained very high. Thus, phospho*enol*pyruvate can be formed from pyruvate and 1 : 3-diphosphoglycerate (1 : 3-diPGA) from PGA in large enough amounts to be amenable for preparative procedures. However, 1 : 3-diPGA is hydrolysed quite rapidly even at neutral pH at 37°. Thus, starting with ATP and catalytic amounts of PGA in the presence of a system which regenerates ATP (e.g., PGA with PGA mutase, enolase and pyruvate kinase or phospho*enol*pyruvate with pyruvate kinase), one can observe an ATPase activity which is quite appreciable. Indeed, it hindered early attempts to demonstrate reversal of glycolysis.

Although there is no evidence that a PGA-dependent ATPase activity is functional in intact cells, it may serve as a model for a selfadjusting metabolic mechanism which would allow for a limited rate of glucose breakdown in Pi-deficient cells.

There follows a brief discussion of a second metabolic feature of the intact ascites cells. It has been known for over 30 years that tumour cells glycolyse aerobically, but there is no adequate explanation for this phenomenon. Straub and his collaborators (Acs *et al.*, 1955) have proposed that hexokinase associated with mitochondria of ascites cells may account for the high glycolysis. Though we have confirmed their finding that a considerable proportion of tumour hexokinase appears in the mitochondrial fraction, we are not

certain that this is not a preparative artifact. Moreover, we could not find appreciable amounts of phosphofructokinase or of the other glycolytic enzymes in the particles. Almost all of their activities were recovered in the supernatant fraction after high-speed centrifugation (Wu and Racker, 1959). As a general phenomenon mitochondrial glycolysis of tumours, though representing the basis of an attractive hypothesis, is in our opinion non-existent.

There are two features of the metabolism of ascites tumour cells which favour an alternative explanation for their high aerobic glycolysis. First of all, the high potential capacity of the glycolytic enzymes, especially the slight excess of G-3-p dehydrogenase and the preponderance of the transphosphorylating enzymes permits the effective competition of glycolysis for ADP and Pi. Secondly, although Pi has been demonstrated to be a limiting factor of glycolysis in ascites cells, the apparent intracellular concentration is rather high in reference to the K_m of Pi for G-3-p dehydrogenase (Wu and Racker, 1959). Even at the lowest Pi concentration observed intracellularly a reconstructed system of glycolysis functions almost maximally. It appears that we are confronted with a problem of availability of intracellular phosphate. Since time does not permit dwelling on this subject matter, it may suffice to propose that (*a*) compartmentation controls the availability of Pi and other intracellular components, and (*b*) compartmentation is less rigid in tumour cells than in "normal" cells. It will be of interest to make comparative studies in tumour and normal cells of compartmentation of the type discussed by Prof. Lynen and also to explore some other known phenomena (Cori, 1956) which indicate compartmentation. It is possible also that a diminished rigidity of the intracellular structure may find an expression in increased penetration of extracellular phosphate and other compounds which enter with difficulty. Some of these possibilities are being investigated at the present time.

Whatever the reason for the increased glycolytic rate of tumour cells may be, we believe that this feature should be of

considerable importance for the physiology of the tumour cell. Since this is not the only feature characteristic of tumour cells, it becomes unimportant that some "normal" cells, such as the leucocytes (which do not divide) also have a high aerobic glycolysis. Whether or not one wishes to accept the thesis that a damaged respiration is the primary lesion which leads to increased glycolysis, one can only be impressed by the universality of the high aerobic tumour glycolysis, which must exert a decisive effect on all the other aspects of intermediary metabolism.

Summary

A systematic investigation of the pacemaker of glycolysis in ascites tumour cells revealed that the availability of inorganic phosphate limits the rate of both lactic acid production and glucose uptake. The controlling effect of inorganic phosphate on glucose uptake is indirect due to limitation of the glycolytic ATP regeneration, since ATP formed during oxidative phosphorylation is not as efficiently utilized for glucose phosphorylation. The Pasteur effect can be interpreted in terms of the decreased availability of inorganic phosphate under aerobic conditions and the subsequent diminution of glycolytic production of ATP.

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DISCUSSION

Lehninger: Is acyl phosphatase present in the ascites cell extracts? Harary has proposed a mechanism for dephosphorylation of ATP through 1 : 3-diphosphoglycerate (Harary, I. (1957). *Biochim. biophys. Acta*, **26**, 434).

Racker: Krimsky in our laboratory has studied the hydrolysis of 1 : 3-diphosphoglycerate. The acyl phosphatase described by Prof. Lipmann many years ago was found to cleave 1 : 3-diphosphoglycerate. In fact, 1 : 3-diphosphoglycerate is so labile that, when a high enough concentration of it is maintained, the rate of non-enzymic hydrolysis is quite rapid. In the presence of 3-phosphoglycerate (PGA), PGA kinase and an ATP-regenerating system, the spontaneous hydrolysis of 1 : 3-diphosphoglycerate is quite appreciable so that it gives the appearance of the presence of an active ATPase which is dependent on PGA.

One can readily demonstrate in extracts of ascites tumour cells a PGA-activated "ATPase activity". Whether the observed rate of phosphate liberation is entirely due to spontaneous hydrolysis of 1 : 3-diphosphoglycerate, or whether other mechanisms contribute (e.g. specific acyl phosphatases) has not been established. It may be added that in addition to the heat-stable enzyme, skeletal muscle contains a heat-labile phosphatase with somewhat different properties. A heat-labile enzyme was also observed by Harary to be present in liver.

Chance: This model is largely based upon experiments with ascites tumour cells in which we have demonstrated three different metabolic events following glucose addition (Chance, B., and Hess, B. (1959). *Science*, in press): (1) the activation of respiratory and phosphorylative activity upon adding glucose; (2) an inhibition of intracellular ATP utilization by a lowering of the intracellular ATP level; (3) an inhibition of both respiration and glucose utilization when the added glucose exceeds the available ATP store. The latter regulation is related to, but more intense than, that which is recognized as the Crabtree effect. It is the purpose of the model to demonstrate whether or not these regulations can be manifestations of the same process of metabolic control.

The model contains four basic functions as indicated by the four main blocks of Fig. 1A. First, phosphorylation of glucose; second, glycolytic phosphorylations of ADP; third, oxidative phosphorylations of ADP; fourth, ATP utilization. In these experiments, the glucose concentration is initially zero, but the system contains a pyruvate store, an inorganic phosphate store and two ATP stores, one associated with the glucose phosphorylation enzymes and the other associated with the oxidative phosphorylative system. Respiratory metabolism is limited by the rate of ATP utilization, the rate of which is set not only by the needs of the cell for synthesis, transport, etc., but also by the rate at which ATP is made available from store II to store I (the assignment of endogenous ATP utilization to ATP directly expendable from store I is not wholly arbitrary since regulation 2 mentioned above appears to require that this be so).

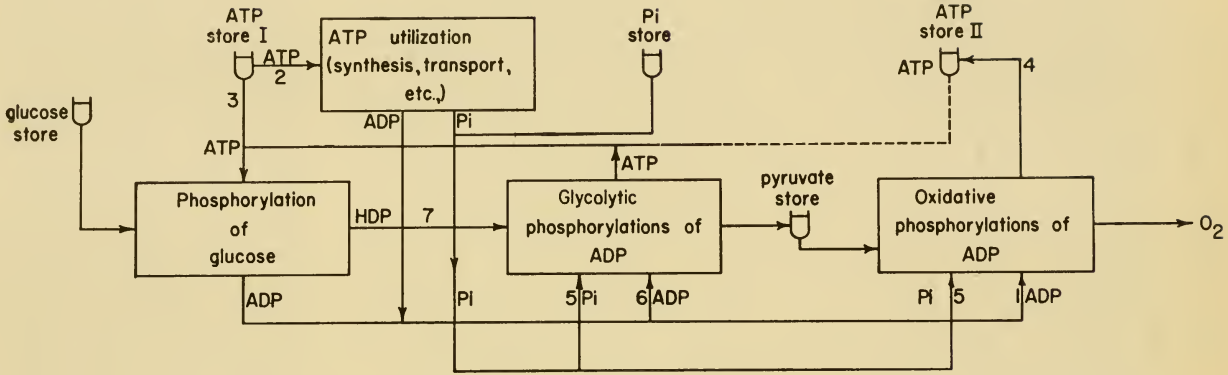

FIG. 1A (Chance). (Expt. no. MD–65)

Table I gives the chemical equations which have been represented together with the initial concentrations and reaction velocity constants.

Table I (Chance)

(Expt. no. ME–62) Chemical equations for interactions of glucose-oxygen pathways.

Phosphorylation of glucose	1.	$0\text{-}4\times10^{3}$ GLU	+	10^{5} ENZ	3×10^{8} ⟶	ENG - GLU - ENZ
	2.	0 ENG	+	10^{3} ITP	6×10^{4} ⟶	ADP + ENZ + GLP - ENG - ITP
	3.	0 GLP	+	1×10^{4} ETZ	10^{10} ⟶	ETG - GLP - ETZ
	4.	0 ETG	+	10^{3} ITP	6×10^{8} ⟶	GPP + ETZ + ADP - ITP - ETG
	5.	0 GPP			10^{5} ⟶	(2) GAP - GPP
Glycolytic Phosphorylations of ADP	6.	0 GAP	+	10^{4} DPN	10^{8} ⟶	DPH + BGA - GAP - DPN
	7.	0 BGA	+	5×10^{3} PIΔ	10^{7} ⟶	DGA - BGA - PIΔ
	8.	0 DGA	+	10^{4} ADP	10^{8} ⟶	ITP + PGA - DGA - ADP
	9.	0 PGA	+	10^{4} ADP	10^{8} ⟶	ITP + PYR - PGA - ADP
	10.	5×10^{3} PYR	+	10^{4} DPH	5×10^{7} ⟶	LAC + DPN - PYR - DPH
	11.	10^{1} LAC	+	10^{4} DPN	5×10^{4} ⟶	PYR + DPH - LAC - DPN
Oxidative Phosphorylations of ADP	12.	5×10^{3} PYR	+	10^{4} DIN	5×10^{7} ⟶	DIH - DIN - PYR
	13.	10^{4} DIH	+	10^{4} X·I + 1.5×10^{3} OXI	6×10^{11} ⟶	DIN + XSI - OXY - X·I - DIH
	14.	10^{4} XSI	+	10^{4} ADP + 5×10^{3} PIΔ	6×10^{10} ⟶	2TP + X·I - PIΔ - ADP - XSI
ATP Utilization and Transfer	15.	10^{3} 2TP	+	$0\text{-}10^{3}$ DBP	2×10^{6} ⟶	1TP - 2TP
	16.	10^{3} 1TP	+	2×10^{6} PUE	10^{9} ⟶	PPP - 1TP - PUE
	17.	10^{6} PPP			10^{6} ⟶	PUE + ADP + PΔI - PPP

There are four sets of chemical equations corresponding to the four blocks of Fig. 1A above. The chemical equations are written in the three-letter abbreviations that the computer can accept, the reactants

being on the left-hand side and the products being on the right-hand side. Although it is possible to take into account reversibility of these reactions, this is omitted for the sake of simplicity in this preliminary study.

The equations in the category labelled "phosphorylation of glucose" represent the combination of glucose (GLU) concentrations between zero and 4×10^{-3} M with 10^{-5} M hexokinase (ENZ) with a velocity constant of 3×10^{-8} M^{-1} sec^{-1} to form a glucose enzyme intermediate (ENG). Any reactant that disappears in the chemical change must be so indicated on the right-hand side and the reaction product is written ENG — GLU — ENZ. The second step is the reaction of the glucose-enzyme intermediate (ENG) of zero initial concentration with 10^{-3} M ATP available in store I (ITP), with a velocity constant of 6×10^{-4} M^{-1} sec^{-1} to form ADP (which is not compartmented) plus the free enzyme (ENZ) plus glucose-6-phosphate (GLP) together with a notation of those substances which are expended in this reaction (— ENG — ITP). The isomerase step is assumed not to be rate-limiting and GLP is reacted directly with phosphohexokinase (ETZ) to form an intermediate which in equation 4 reacts with ATP to form fructose diphosphate (GPP). In the aldolase reaction (equation 5) the enzyme-substrate intermediate is omitted and a first-order velocity constant of 10^5 $sec.^{-1}$ leads to the formation of two molecules of reaction product. The isomerase step is omitted and it is assumed that all of the dihydroxyacetone phosphate can be made available as glyceraldehyde phosphate (GAP).

In order to reduce the number of equations and to simplify the calculations, enzyme substrate intermediates are omitted in equations 6–15 and, instead, a velocity constant for the second order reaction of the two substrates is included. In the glycolytic phosphorylations of ADP (equation 6), glyceraldehyde phosphate (GAP) (at an initial concentration of zero) reacts with 10^{-4} M DPN (DPN) with a velocity constant of 10^{-8} M^{-1} sec^{-1} to give reduced DPN and an intermediate BGA. For simplicity, the separate steps of GAP and DPN with the enzyme are omitted, and BGA represents the enzyme-substrate-coenzyme complex. In equation 7 the phosphorolysis of this complex with 5×10^{-3} M inorganic phosphate (PIΔ) is represented to have a velocity constant of 10^7. The reaction product, 1 : 3-diphosphoglyceric acid (DGA) then reacts with ADP with 10^{-4} M ADP at a velocity constant of 10^8 to form ATP of store I (ITP) and 3-phosphoglyceric acid (PGA). At this point both the phosphoglyceromutase and enolase steps are assumed not to be rate-limiting steps and the overall reaction from 3-phosphoglyceric acid to pyruvate (PYR) is written as a single step. In equation 9, ATP available to store I is formed (ITP).

Equations 10 and 11 represent the lactate-pyruvate equilibrium and constitute the pyruvate store. Therefore, appropriate initial concentrations of these two substrates are based upon the equilibrium constant. These concentrations together with the initial concentrations of DPNH (DPH) and DPN (DPN) afford a supply of metabolites for the mitochondria.

Reactions occurring within the mitochondrial membrane are indicated by equations 12, 13 and 14, and all of the processes of the citric acid

cycle and the respiratory chain have been condensed to three equations for the sake of simplicity. Equation 12 represents the reaction of pyruvate which is exchangeable between the mitochondrial and the cytoplasmic spaces with the DPN that is enclosed within the mitochondrial membrane (DIN). The DPNH so formed (DIH) is also restricted to the mitochondrial space. Such DPNH reacts with oxygen (OXY) and a low energy of an intermediate in oxidative phosphorylation (X·I) to form oxidized DPN and a common high-energy intermediate in oxidative phosphorylation (XSI). This intermediate can then interact with ADP and phosphate in equation 14 to form ATP in store II (2TP).

In the last functional block, the utilization of ATP is represented by equations 15–17. The last two equations represent the interaction of ATP in store I with phosphate utilization enzymes (PUE) to give an enzyme intermediate PPP which decomposes in a first-order reaction to give ADP and phosphate, which readily activates respiration.

Equation 15 is a most important one since it represents a mechanism by which ATP can be freed from the mitochondrial store (2TP) and made available to the cytoplasmic store (ITP). Experimentally this is observed to be caused by the addition of uncoupling agent such as dibromophenol and dicoumarol. There may be a physiological substance of unknown nature which may be responsible for this. However, we have simply indicated that there may be added a concentration of dibromophenol (DBP) between zero and 10^{-3} M to activate this interchange.

The reaction velocity constants used in this mechanism are rather arbitrarily chosen. In fact, the deciding factor in choosing the reaction velocity constant has often been that the product of the steady state concentrations of the reactions involved and the reaction velocity constant give a metabolic flux between one and ten per second. The latter figure is reasonable in view of our observations of the turnover number of the respiratory enzymes in the intact cell. Thus, inconsistencies between initial concentrations and reaction velocity constants of this mechanism and those of the actual cell may occur. Nevertheless, the kinetic and steady state behaviour of the system appears to be a reasonably good representation of that of the intact cell. As more accurate data on reaction rates and concentrations become available the computer data can, of course, readily be amended.

The programming of the digital computer (Univac I) is quite outside the scope of this talk and will be reported elsewhere by Garfinkel, Higgins and Rutledge who have developed the equation-solving process. In brief, the computer recognizes the names of the chemicals, the nature of their interactions, the reaction velocity constants, the initial concentration and maximal concentrations, and the stoichiometric ratios.

Fig. 1B shows a graph of the kinetics of some of the chemicals in the system of Table I. This graph is computed from tabular data made available by the digital computer and is typewritten on a high speed printer in the form illustrated. The ordinates are concentrations and the abcissae are computer time intervals (typewritten at bottom). Only the main features of the graph will be indicated here.

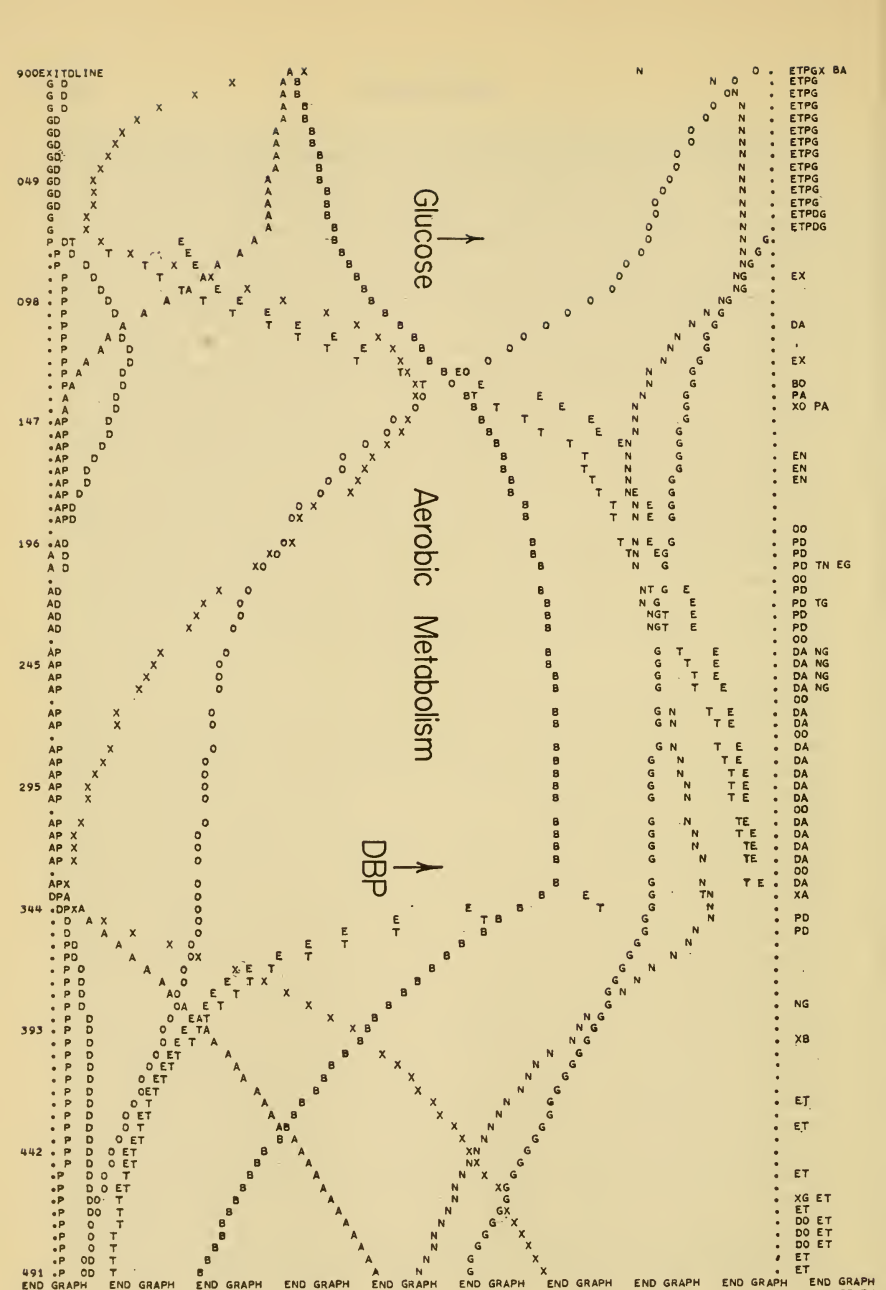

FIG. 1B (Chance) (Expt. no. DG1)

As the computer solution is initiated, the rate-controlling intermediate of respiration X·I (X) is rapidly expended with the corresponding utilization of oxygen (O). The initially low ADP concentration (D) falls towards zero and oxygen utilization slackens appreciably. As a result of the expenditure of X·I the ATP store in the mitochondria (2TP) (B) has increased slightly. The cells are then in state 4 which is characterized by a low respiratory rate set by the low rate of utilization of endogenous ATP (equations 16 and 17). The system is now in the proper condition for the demonstration of the metabolic transient caused by glucose addition and this is marked by the arrow and by the appearance of the glucose concentration at the top of the trace (G). The first effect is an abrupt decrease of the ATP store available for glucose phosphorylation (ITP) (A) and this substance continues to diminish. Simultaneously, the concentrations of intermediates in glucose phosphorylation rise, with a jump in the case of the hexokinase intermediate ENG (E) and smoothly in the case of the phosphohexokinase intermediate ETG (T). With a short delay, DPN (N) is reduced by the formation of glyceraldehyde phosphate (GAP).

At the mitochondria, the rise of ADP concentration (D) leads to an abrupt increase of the X·I intermediate (X) and hence to a rapid stimulation of the utilization of oxygen (O).

Since the store of ATP [ITP(A)] available to glucose phosphorylation is nearly completely expended, the rate of glucose utilization falls to a very low value. The ADP made available by the rapid rush of glucose utilization begins to fall and causes a reduction of the concentration of the X·I compound (X). Thus, at a computation time between 295 and 340 the oxygen (O) and glucose (G) metabolism comes very nearly to a halt, and the ATP store II (2TP) (B) reaches a high concentration.

At computation time, addition of DBP (see Fig. 1B) allows ATP from mitochondrial store II (2TP) to be transferred to the cytoplasmic store I (ITP). At this moment there is a rapid rise of ATP in store I (A) together with a rapid drop in intermediates T and E of glucose phosphorylation and a reduction of pyridine nucleotide (N). Glucose utilization is stimulated and the ADP formed stimulates the respiration. In the ensuing computation intervals (344–491) the metabolism of the cell goes through a transition which might well be termed a Pasteur effect. The computation was stopped at this time, and the aerobic-anaerobic transition is not shown. Then the cessation of ATP synthesis in the mitochondria would be compensated by the glycolytic production of ATP.

In this very preliminary digital computer analysis of interaction of glycolysis and respiration, we have employed a very simplified system for all the chemical reactions. In addition, we have been forced to make arbitrary choices of concentrations and velocity constants. Nevertheless, the system demonstrates important metabolic regulations which we have been able to study experimentally in ascites tumour suspensions. First, it shows the acceleration of respiration caused by glucose addition together with a subsequent inhibition of both glucose and oxygen metabolism. Lastly, it shows a type of Pasteur response in

which aerobic glucose utilization is rapidly accelerated by a greater availability of ATP in the cytoplasm. As more accurate data become available on reaction velocity constants and enzyme concentrations, this minimal hypothesis may be greatly further refined and may be used to point the way to new experimental tests of theories on metabolic control.

Siekevitz: It has recently been found that the mitochondria cannot store ATP (Siekevitz, P., and Potter, V. R. (1955). *J. biol. Chem.*, **215**, 237; Pressman, B. C. (1958). *J. biol. Chem.*, **232**, 947). During oxidative phosphorylation it is made and it goes out, and it is not stored.

Chance: Mitochondria isolated from the liver contain ATP, which does not go directly to hexokinase; apparently it is stored at a certain point and is not available.

Siekevitz: As more is made it is put out.

Chance: They are isolated and are not the same as in the cell.

Bartley: Dr. Racker, does the extra phosphate that is taken up when you increase the external phosphate mix with the pool of internal phosphate in the cell?

Racker: The measurements of transport by ^{32}P determinations and by chemical analysis of intracellular phosphates have been in reasonably good agreement (making reasonable corrections, of course). The phosphate which is thus taken up must mix to some extent with the intracellular pool of inorganic phosphate which is available for esterification. The distribution of ^{32}P into the various organic phosphate fractions has been explored and it seems that under our experimental conditions the incorporation into phosphate esters is quite rapid.

Bartley: If you use radioactive phosphate in the early stages, this will give you the size of the effective phosphate pool.

Racker: Our kinetic studies of the early stages of phosphate transport have not been too extensive but there are already indications that the course of ATP labelling does not quite follow that of the intracellular Pi. This type of experiment may give us some further clues to the problem of compartmentation in the ascites tumour cells.

Greville: I understand that the Pasteur effect in tumour cells results from interaction between respiring mitochondria and the glycolytic system. Now in rabbit skeletal muscle, for instance, where there are practically no mitochondria, the mitochondria can have very little influence on the glycolysis. I wonder whether in the ascites tumour cells and other tumour cells there is a low content of mitochondria, or whether the mitochondria have a light load of respiratory enzymes; either of these might account for the aerobic glycolysis.

Racker: Is respiration really low? The Q_{O_2} of ascites tumour cells, even according to Warburg, is 7, which is quite respectable. Weinhouse has obtained even higher values. The question of whether tumour cells have a large or small Pasteur effect seems to depend on the point of view. Although Warburg has maintained that the relative Pasteur effect as calculated on a percentage basis is smaller in tumour tissues, Weinhouse has pointed out that the absolute Pasteur effect, which is the amount of lactic acid suppressed, is actually even greater in tumour

cells as compared to normal cells. This reminds me of the story of what happened on one June 21st: One young lady whose nationality shall remain undisclosed turned to the young man and said, "Do you realize that this is the longest day in the year?", while the French girl turned to her young man and said, "Do you realize that this is the shortest night of the year?"

To come back to serious matters, I would like to point out that irrespective of which view one subscribes to, the major fact is that tumour cells produce a large amount of lactic acid under aerobic conditions. I feel that this fact by itself must have a very pronounced influence on the whole metabolic machinery of the cell. I would not be surprised if these changes in intracellular pH have a tremendous effect on the entire metabolism and physiology of the cell.

Greville: This, of course, is a 30-year-old controversy; but, in general, non-malignant cells have an anaerobic glycolysis more commensurate with their respiration than do tumour cells. Is there not a basic disparity between the anaerobic glycolysis and respiration in most tumour cells? In other words, in malignant cells a small respiration is trying to cope with a big anaerobic glycolysis.

Racker: I believe that this is true for most tissues, but what about brain tissue and yeast cells which have a very high anaerobic glycolysis?

McIlwain: From this point of view, certain cerebral tumours do follow the pattern of tumours from other tissues. They show greater aerobic glycolysis than do normal cerebral tissues.

Chance: The ascites tumour cell has the same cytochrome content as the liver cell.

Lehninger: Dr. Racker, on the question of identity of the inorganic phosphate that you measure, is that really inorganic phosphate? Some of it might be a very labile phosphate ester.

Racker: Prof. Lynen pointed out many years ago that the chemical analysis of the intracellular inorganic phosphate might include some acid-labile, high-energy phosphate compounds. We have not been able to detect, at least in ascites tumour cells, significant amounts of acyl phosphate. We therefore feel at the present time that we actually measure intracellular inorganic phosphate by chemical analysis. It just appears from the metabolic studies that some of this inorganic phosphate is not available for glycolysis.

Lynen: That is my idea too. Dr. Holzer, in my laboratory, tried to detect acyl phosphate in intact yeast cells but could not find any.

Hess: I think that a certain amount of the inorganic phosphate is readily available in the mitochondria for respiration. As I have already shown (Fig. 1, p. 126), a certain amount of inorganic phosphate is taken up immediately in stoichiometric relationship to the oxygen uptake at the same time after addition of glucose. It can be related to the P/O ratio and is also in a relationship of 1/1 to the glucose consumed. This store of inorganic phosphate is definitely limited by a minimal concentration of approximately 6 μM phosphate/g. wet weight of ascites tumour cells. There might be a difference between your experimental conditions and ours. In this connexion, I would point out that in some of these

experiments where we do not add glucose in excess, the ATP in the store near the hexokinase is not depleted. I understand that your glucose concentrations are so high that you have no ATP left in this type of store.

Racker: The glucose concentrations which we used were quite high, of the order of the physiological blood concentration. But, as you can see from our data, at no time was the ATP depleted; in fact, the addition of glucose increased ATP concentration rather than decreased it. The initial dip in the graph, which is due to ADP formation, is only very short-lived.

Hess: We have also demonstrated this drop. The fact that there is a constant minimum level of ATP per cell, together with other data, led us to assume the occurrence of various cellular ATP pools; in fact, it can be taken as evidence for it (see Hess, B., and Chance, B. (1959), in preparation; Chance, B., and Hess, B. (1959). *Science*, in press). Thus, this minimum ATP is in the mitochondria and not at the hexokinase.

Racker: This is exactly the point I am trying to make.

Hess: If one adds a very small amount of glucose, as we do, in the range of 0·15 m-molar and titrates just a small amount of the ATP at the site of the hexokinase, one does not see a large drop in the ATP level. It should be emphasized that in simulation of the physiological state we have to add a small amount of glucose, since the abdominal cavity contains very low amounts of glucose. We must assume that under physiological conditions the cells are idling between different states. One of these states is defined by low-level glucose which penetrates into the cell and just controls the respiratory rate, keeping it at its lowest level.

Racker: I cannot quite agree on this point. I don't think we know much about the *in vivo* rate of glucose utilization of ascites tumour cells within the peritoneal cavity. I don't think we should be too impressed by the fact that we don't find very much glucose when we analyse the intraperitoneal fluid. As long as we have no information on the rate of glucose delivery from the blood stream, we cannot estimate the rate of its utilization. Similarly, if we find no oxygen in the ascites fluid, we should not conclude that the ascites tumour cells do not respire. It is quite possible that the rate of diffusion of glucose or oxygen is slower than the rate of utilization. I would also like to point out that measurements of glucose concentration in ascites fluid may be misleading because of the time required for sampling. We are dealing with a heavy suspension of actively metabolizing cells which can utilize a respectable amount of glucose in a matter of a few seconds.

Would you want to say that the intraperitoneal steady state concentration of glucose is below the K_m of hexosamine, which in ascites tumour cells is below 10^{-4} M?

Chance: Dr. Hess pointed out that it was the rate of production of ATP from the glucose hexokinase system, which was setting the pace of respiration.

Racker: I still do not see what that tells us about the rate of glucose utilization.

Chance: That means that the rate of glucose utilization is probably proportional to the respiration.

Hess: We have never seen this high glucose concentration (10 mM) in the abdomen; we might see it on the first day after inoculation, but then the glucose concentration drops down.

Lipmann: I have been worried by the fact that anaerobic cells, e.g. yeast, can be inhibited in their growth by azide and by DNP. If we distinguish as sharply as we do here, between aerobic energy supply and glycolytic energy supply, perhaps we should consider that there might be a common pathway. Both of them seem to have to be pushed through. Otherwise I cannot understand why these inhibitors act on the glycolytic system or on the energy coming out of the cell.

Racker: What about stimulation of ATPase?

Lipmann: I do not believe in that so much; ATPase is something which we have constructed and which does not exist.

Lynen: I would agree with Prof. Lipmann on that point. From our experiments with yeast cells we also deduced that ATPase in the classical sense does not exist. ATPase is just the sum of all the processes where ATP is split into ADP and phosphate, or AMP and pyrophosphate.

Racker: We have observed repeatedly that dinitrophenol stimulates anaerobic glycolysis in ascites cells. Prof. Lynen, did you not report experiments on yeast in collaboration with Dr. Koenigsberger (1951, *loc. cit.*), in which you observed an effect of DNP on intracellular dephosphorylations in the presence of cyanide?

Lynen: We measured the dephosphorylation rate by adding large amounts of KCN to metabolizing cells, thereby initiating the formation of inorganic phosphate, which can be measured at intervals. When we added DNP and KCN together, no change in dephosphorylation rate was observed.

RÔLE OF TRIPHOSPHOPYRIDINE NUCLEOTIDE IN THE REGULATION OF GLYCOLYSIS IN A CELL-FREE PREPARATION*

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Introduction

IT was previously reported that the addition of liver mitochondria to a glycolytic system consisting of the supernatant fraction of brain and tumour homogenates decreases both the glucose uptake and the lactic acid production (Aisenberg, Reinafarje and Potter, 1957; Aisenberg and Potter, 1957). These facts were interpreted as a Pasteur effect produced by respiring mitochondria. The mitochondria obtained from certain tumours did not exhibit such effect. A similar behaviour has been shown recently with mitochondria obtained from Ehrlich ascites tumour (Tiedemann and Born, 1958). Later on, it was demonstrated that the mechanisms concerned with the oxidation of reduced triphosphopyridine nucleotide (TPNH) are decreased or absent in Novikoff hepatoma, as well as in some other tumours (Reinafarje and Potter, 1957).

Since it is a well established fact that tumours possess a characteristically high aerobic glycolysis (Warburg, 1930), it seemed worthwhile to consider the possibility that oxidized triphosphopyridine nucleotide (TPN) could exert a control on glycolysis.

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The experimental evidence indicates that triphosphopyridine nucleotide may inhibit glycolysis in a brain supernatant system, under certain well defined conditions.

Material and Methods

The glycolytic system consisted of the supernatant of a 10 per cent homogenate of rat brain in 0·15 M-KCl [with 0·1 volume of 0·1 M-tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7·4 in some experiments], obtained by centrifugation at 5,000 ***g*** for 10 minutes (S_2) or at 105,000 ***g*** during 60 minutes (S_3); 0·5 ml. of any of these preparations was added to a reaction mixture containing ATP, ADP, DPN, Mg, inorganic phosphate and Tris or bicarbonate buffers. The total volume was 1·6 ml. The exact composition of the incubation medium varied somewhat in the course of the experiments and will be indicated in each table or figure. The system was incubated at 37° for 30 minutes, with air (or oxygen with 5 per cent CO_2) as the gas phase.

Occasionally, fresh or aged mitochondria were added to the system. Mitochondria were prepared from a 10 per cent rat liver homogenate in 0·25 M sucrose, by centrifugation first at 600 ***g*** and then at 5,000 ***g*** (Schneider and Hogeboom, 1950). Fresh mitochondria were washed twice with sucrose and twice or three times with isotonic KCl before use. Aged mitochondria were obtained by incubating the mitochondria, after the second washing with sucrose, in glass-distilled water during two hours at room temperature, with shaking (Reinafarje and Potter, 1957). They were then washed with isotonic KCl. The final suspension of the mitochondria was made in KCl (or KCl-Tris), at a concentration of 50 or 100 mg.-equivalents of liver per 0·1 ml.

Materials—The nucleotides used were commercial products obtained from Sigma Chemical Company, St. Louis, Missouri, or Pabst Laboratories, Milwaukee, Wisconsin. TPN was purified from metal contaminants by passing it through Dowex-50. About 100 mg. of the commercial preparation was

diluted in 2 ml. of water and passed in the cold through a small column (2 cm. in length, 0·8 cm. in diameter) of Dowex-50 in the hydrogen form. The nucleotide was retained, possibly not as the consequence of an ionic exchange, but merely retained in solution in the intergranular water. The elution from the column was done with small portions of water (0·5 ml.). The fractions were assayed enzymically. The recovery was practically complete. TPNH was also purified from possible metal contaminants by passing it through Dowex-50 in the sodium form. The spectrophotometric studies showed no alteration of the nucleotide with this procedure. Glucose-6-phosphate (G-6-P) was the crystalline potassium salt from Sigma. Fructose-1 : 6-diphosphate (F-di-P) and pyruvic acid were purified preparations provided by Dr. G. A. LePage. 6-Phosphogluconate (6-PG) was either a Sigma product or one prepared by Dr. C. Scholtissek by the procedure of Robison and King (1931).

Analytical procedures—Glucose was determined in barium hydroxide–zinc sulphate filtrate (Somogyi, 1945*b*) with the reagents of Somogyi (1945*a*) and Nelson (1944). In some experiments, glucose oxidase was used for this purpose. Lactic acid was determined by the procedure of Barker and Summerson (1941), with some of the modifications introduced by Hullin and Noble (1954), and inorganic phosphate by the method of Fiske and Subbarow (1925) as used by Lohmann and Jendrassik (1926). TPN was determined in an aliquot that was boiled in acid to destroy TPNH (Glock and McLean, 1955). After neutralization, *iso*citric dehydrogenase and *iso*-citric acid were added and readings at 340 mμ were taken in the Beckman-DU spectrophotometer until a plateau was reached. The difference between the plateau and the initial reading was converted into μmole of TPN using the absorption coefficient of $6 \cdot 22 \times 10^3$ (Horecker and Kornberg, 1948). An approximate estimation of TPNH was considered to be the reading at 340 mμ, corrected by the appropriate control, accepting that all the diphosphopyridine nucleotide (DPN) of the system was in the oxidized form (Aisenberg and Potter,

1957). 6-PG was determined in a neutralized perchloric filtrate, measuring the height of the plateau obtained after treatment with an excess of TPN and 6-PG dehydrogenase preparation deprived of G-6-P dehydrogenase activity (Glock and McLean, 1953).

G-6-P and 6-PG dehydrogenases were determined by either one of the two procedures of Glock and McLean (1953). As a measure of hexokinase, the rate of reduction of TPN by glucose plus ATP was considered convenient, provided that the preparation had an excess of G-6-P dehydrogenase. The reduction of DPN, in the presence of fluoride and arsenate, was used to measure some parts of the glycolytic chain, employing different substrates, such as glucose, hexose phosphates or triose phosphates, according to the purpose of the assay. The detailed composition of the reaction mixtures will be indicated during the presentation of the results.

Results

General conditions

The S_2 preparation of rat brain homogenate (supernatant plus microsomes) can convert glucose into lactic acid at a higher rate than the supernatant alone (S_3). Notwithstanding, most of the experiments were performed with the latter preparation, which was considered simpler than the former and thus more convenient for our purposes.

The system does not require that F-di-P be added, as has been done in similar systems by LePage (1948) and by Aisenberg, Reinafarje and Potter (1957). An adequate amount of adenosine triphosphate (ATP) must be provided in the reaction mixture.

The reaction is markedly dependent on the pH of the medium (LePage, 1948), as can be seen in Fig. 1, which also shows the influence of pH on the effect of TPN on glycolysis.

Effect of mitochondria—The addition of fresh mitochondria decreased glycolysis of brain supernatant, thus confirming previous findings (Aisenberg, Reinafarje and Potter, 1957;

Aisenberg and Potter, 1957; Gatt, Krimsky and Racker, 1956; Tiedemann and Born, 1958). The action of fresh mitochondria is very complex, because they contribute many enzyme systems that may interfere with glycolysis through different mechanisms (Aisenberg and Potter, 1957). In addition to their capacity to oxidize the pyridine coenzymes

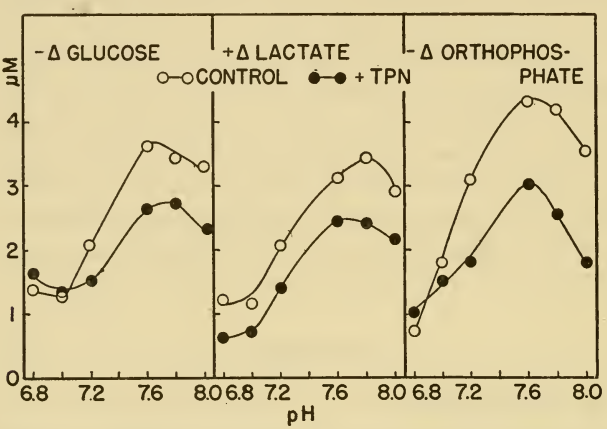

FIG. 1. Influence of pH on glycolysis and on the effect of TPN. Each flask contained in a 1·6-ml. volume, 0·5 ml. brain-S_3, 7·5 μM glucose, 2 μM ATP, 1 μM DPN, 60 μM nicotinamide, 6 μM-$MgCl_2$, 15 μM potassium phosphate at the required pH; potassium bicarbonate in concentration to give the required pH when equilibrated with 95 per cent O_2–5 per cent CO_2, 3 μM TPN when indicated. 0·15 M-KCl to maintain isotonicity.

and pyruvate and to carry out oxidative phosphorylation, they contain variable amounts of coenzymes and may be often contaminated in the case of the liver with G-6-Pase from the microsome fraction, which obscures the interpretation of some experimental data. For these reasons, aged liver mitochondria were preferred for further studies. They are depleted of coenzymes (Siekevitz and Potter, 1955), but are able to oxidize DPNH through the DPN-cytochrome *c* reductase and TPNH through transhydrogenase and the preceding enzyme (Reinafarje and Potter, 1957). On the other hand, they are presum-

Table I

PHOSPHATASE ACTIVITIES OF LIVER MITOCHONDRIA

Expt.	*Mitochondria* (200 *mg.-equiv.*)	*Substrate*	*P_i liberated* (μM/10 *minutes*)
1	Fresh	G-6-P	1·36
2	Aged	G-6-P	0·17
		ATP	1·62

Each flask contained Tris-buffer pH 7·4; 3 μM of substrate; 3 μM-$MgCl_2$ in a total volume of 1·0 ml. P = inorganic phosphate.

ably unable to oxidize pyruvate or to bring about oxidative phosphorylation (Siekevitz and Potter, 1955). Their G-6-Pase contamination is weak or absent, but they have a considerable

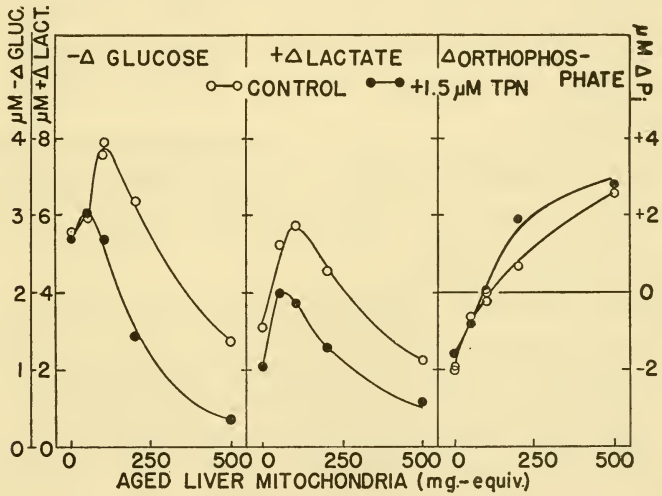

FIG. 2. Influence of variable amounts of aged mitochondria and of TPN on glycolysis. Each flask contained in a 1·6-ml. volume, 0·5 ml. brain-S_3, variable amounts of mitochondria as indicated, 7·5 μM glucose, 1·5 μM ATP, 0·5 μM DPN, 60 μM nicotinamide, 6 μM-$MgCl_2$, 10 μM potassium phosphate pH 7·4, 1·5 μM TPN when indicated. 0·15 M-KCl to maintain isotonicity.

ATPase activity (Table I). The addition of aged liver mitochondria modifies the rate of glycolysis by the brain supernatant, as shown in Fig. 2, which also presents the effect of

TPN. The effect of TPN will be discussed in a later section. The effect of mitochondria could be related to the ATPase activity of the preparation. When small amounts of mitochondria were added to the glycolytic system, a stimulation of glycolysis coincident with less esterification of inorganic phosphate was observed. This is considered as an indication that the additional ATPase was enough to permit the maintenance of the orthophosphate, and to provide more phosphate acceptor (ADP), which could be the limiting factor in the overall process. It seems that the latter effect is the more important, because the concentration of inorganic phosphate in the medium is quite in excess of the requirements. Furthermore, the addition of ADP to the brain system without mitochondria increased glycolysis. ADP also raised the fraction of glucose consumed that appears as lactate, ar effect that was similarly observed with the addition ot mitochondria.

When increasing amounts of mitochondria are added, glycolysis diminishes and there is a net release of inorganic phosphate. It appears that the breakdown of ATP decreases the useful level of this compound in the medium, and thus reduces glucose utilization.

Effect of inorganic phosphate—The great sensitivity of the system to inorganic phosphate was observed in spectrophotometric studies. In these experiments, the reduction of DPN was followed under certain conditions, using glucose or G-6-P as a substrate. This process is strictly dependent on a concentration of inorganic phosphate that exceeds the stoichiometric requirements for the oxidation of 3-phospho-D-glyceraldehyde, probably owing to the formation of fluorophosphate (Fig. 3). In a similar system, DPNH may be oxidized, which is interpreted as the reduction of dihydroxyacetone phosphate to α-glycerophosphate. When F-di-P is used as the initial substrate the oxidation of DPNH is independent of the concentration of inorganic phosphate, as has to be assumed from the knowledge about the mechanism of the particular reactions that participate. However, if glucose or

G-6-P is the starting substrate, the system becomes dependent on inorganic phosphate concentration, as is shown in Fig. 4, and arsenate could replace phosphate. This unexpected situation conforms to the conclusion that in a multi-enzyme system a sequence of reactions may be interrupted by a block in a related reaction, the oxidation of 3-phospho-D-glyceraldehyde in the case we are dealing with, depending

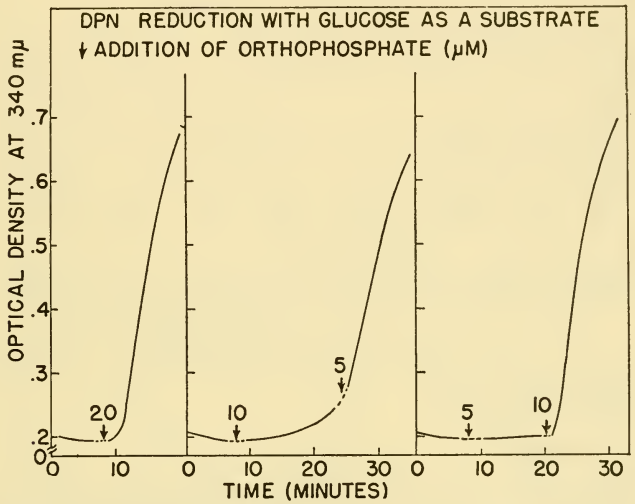

FIG. 3. Influence of phosphate concentration on the reduction of DPN by brain supernatant. Each Beckman cuvette contained in a 3·0-ml. volume, 0·4 ml. brain-S_3, 12 μM glucose, 9 μM ATP, 180 μM NaF, 6 μM DPN, 6 μM-$MgCl_2$, variable amounts of potassium phosphate when indicated, 40 μM Tris buffer pH 7·46. 0·15 M-KCl to complete volume.

on the equilibrium constants and the Michaelis constants of the systems that interact. We interpret our finding as the consequence of a block in the splitting of F-di-P, when production of F-di-P is small, due to an accumulation of a very small amount of 3-phospho-D-glyceraldehyde produced by the absence of inorganic phosphate. The interpretation requires a low affinity of the α-glycerophosphate dehydrogenase for its oxidized substrate as compared with the affinity of aldolase

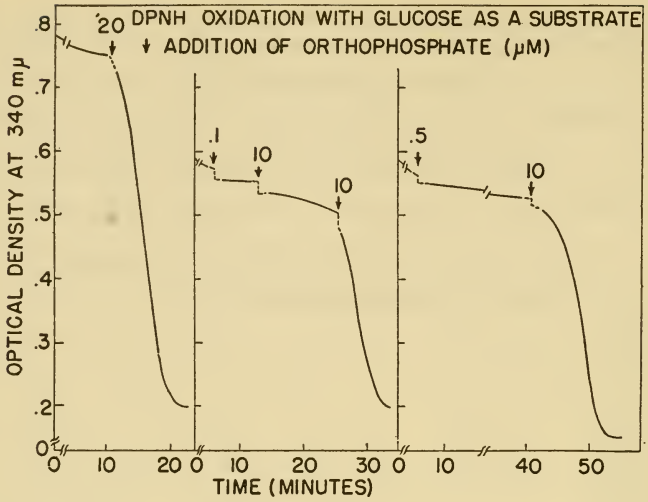

FIG. 4. Influence of phosphate concentration on the oxidation of DPNH by brain supernatant. The composition of the medium was as in Fig. 3, but DPN was replaced by DPNH.

for the triose phosphates. Further studies on this phenomenon were not carried out.

Effect of TPN

It was observed that TPN inhibited glycolysis produced by the S_2 or S_3 preparations from rat brain. The effect could be shown on glucose disappearance, lactic acid production and orthophosphate uptake. In general, the effect on glucose was less reproducible and of less magnitude than that on lactate, and sometimes an increased glucose uptake was observed. The TPN effect depends on the pH of the medium, as illustrated in Fig. 1.

Addition of mitochondria—The addition of fresh mitochondria enhanced the inhibition produced by TPN. Usually the inhibitory effect produced by the simultaneous addition of TPN and mitochondria was greater than that produced by each component separately (Table II). In the presence of aged mitochondria the effect of TPN was also much greater

Table II

INFLUENCE OF TPN AND FRESH MITOCHONDRIA ON GLYCOLYSIS BY A BRAIN-S_2 PREPARATION

Additions	$-\Delta$*glucose* µM	$+\Delta$*lactate* µM
None	3·55	3·02
TPN (1 µM)	2·70	1·88
Mitochondria (200 mg.-equiv.)	2·68	1·37
TPN + mitochondria	1·20	−0·05

Composition of the medium as in Fig. 2.

and it was evident even when the action of TPN alone was slight or absent (Fig. 2).

TPN is slowly reduced by the brain supernatant, but it is maintained in the oxidized form when mitochondria are present (Fig. 5). These experiments seemed to indicate the

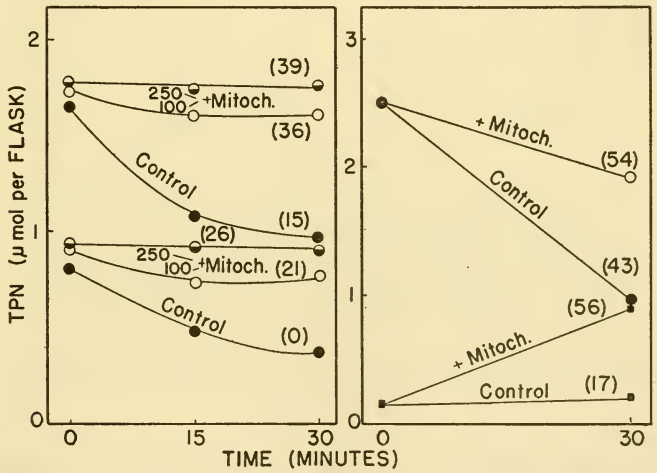

FIG. 5. Effect of mitochondria on the level of oxidation of triphosphopyridine nucleotides incubated with brain supernatant. The composition of the medium was as in Fig. 2 but 0·1 µM cytochrome c was added. TPN was initially present in the experiment of graph A, and TPN (●) or TPNH (■) in that of graph B. The figures in parentheses correspond to the percentage inhibition of glycolysis. The amount of mitochondria is given as mg.-equiv. per flask and refers to the mitochondria obtained from the indicated fresh weight of liver.

importance of the level of oxidation of the TPN on the magnitude of its effect.

Effect of impurities—With the spectrophotometric method it was shown that commercial TPN inhibited the glycolytic chain at different levels. When the reduction of TPN by

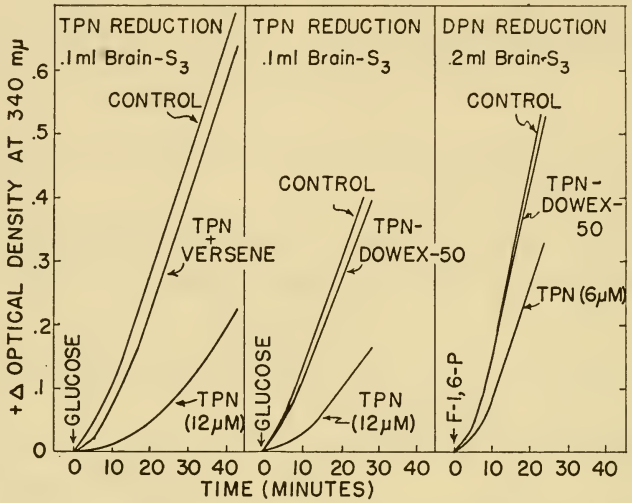

FIG. 6. Influence of ethylenediaminetetra-acetic acid and of treatment with Dowex-50 on the effect of TPN on some reactions of the glycolytic chain. In the *left* and *central* experiments the composition of the reaction mixture was as follows: 0·1 ml. brain supernatant, 100 μM Tris buffer pH 7·46, 12 μM glucose, 6 μM ATP, 12 μM-$MgCl_2$. In the control vessels 1·2 μM TPN was present. 1 μM ethylenediaminetetra-acetic acetate was added when indicated. In the experiment at the *right*, the 3·0 ml. contained: 0·2 ml. of brain supernatant, 5 μM F-di-P, 100 μM Tris, 70 μM sodium arsenate, 180 μM-NaF, 6 μM DPN, and 6 μM-$MgCl_2$.

G-6-P was studied, it was shown that there was not any influence of the level of TPN on the rate of its reduction. But, when glucose was used as the initial substrate, the increasing concentration of TPN increased the lag phase and decreased the slope of the line representing the reduction of TPN (Fig. 6). As the G-6-P dehydrogenase capacity of the brain supernatant exceeds that of hexokinase, and as the

former is not affected by the concentration of TPN, these facts were considered as an indication of an inhibition of hexokinase by TPN. In another system, in which F-di-P was used to reduce DPN, the addition of TPN also produced an inhibition (Fig. 6). The inhibitions were of the non-competitive type, as shown by the fact that the TPN effect depended markedly on the amount of brain preparation used. This is

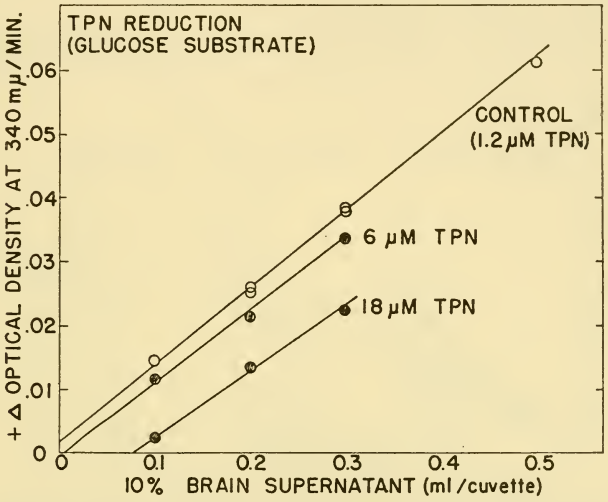

FIG. 7. Titration of the brain enzyme system by commercial TPN. The composition of the medium was similar to that used in Fig. 6 (left and central graphs).

illustrated in Fig. 7 for the system that measures hexokinase activity.

The inhibition of the two different systems could be prevented by the addition of cysteine or ethylenediaminetetraacetate, and also if the TPN was previously passed through a Dowex-50 column, as shown in Fig. 6. These facts strongly suggested that there was a heavy metal contaminating the commercial preparations of TPN, that could explain the inhibition of glycolysis. However, the effects observed in the spectrophotometric studies were obtained with much higher

concentrations of TPN than those used in the complete glycolytic system. In Fig. 8 the dashed line indicates the inhibition of hexokinase that one would expect with the amount of supernatant used in the complete system. At the concentration usually employed in this kind of experiment, the percentage of inhibition would be negligible. The possible

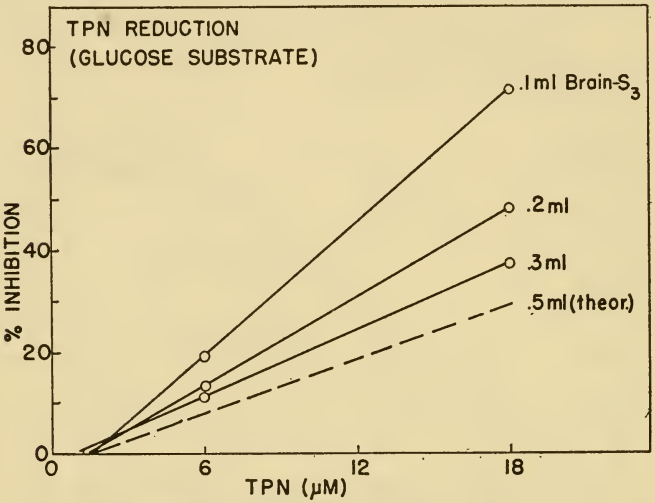

FIG. 8. Influence of the amount of brain supernatant on the inhibition of hexokinase activity by TPN. The data are taken from the same experiment of Fig. 7. The dash line indicates the expected inhibition if 0·5 ml. is used, calculated by extrapolation.

metal contamination would not explain, on the other hand, the influence of mitochondria on the effect of TPN. Nevertheless, in further experiments with the glycolytic system the purified preparation of TPN was always used, with results absolutely comparable to those obtained with the non-purified form, when glucose and lactic acid were determined.

The general conclusion of these experiments was that TPN did not inhibit directly any of the enzymic reactions analysed, but could inhibit indirectly the overall process of glycolysis, an effect that was enhanced by the presence of mitochondria.

Effect of TPNH

If TPN exerted a direct inhibition on glycolysis to bring about a Pasteur effect, it might be expected that TPNH would not have a similar effect. The experimental data showed, however, that TPNH inhibited glycolysis in this system. As with TPN, its effect was less constant on glucose uptake and sometimes an increase was observed. The effect was, in general, less than that of TPN.

Effect of degradative products—The effect of TPNH was complicated by the fact that commercial preparations often have

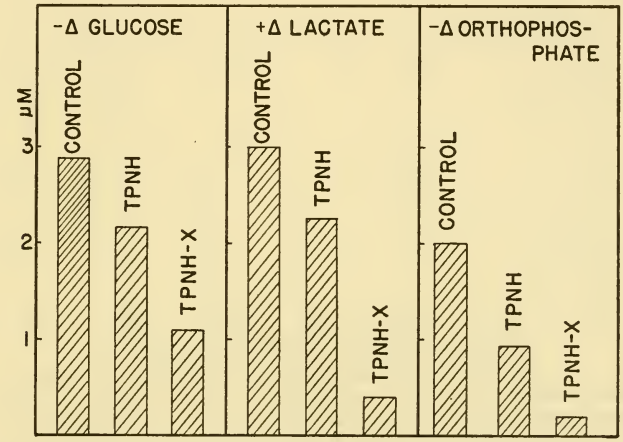

FIG. 9. Effect of a degradative product of TPNH on glycolysis. The composition of the medium was similar to that of Fig. 1. 3 μM TPNH or the equivalent amount of the degradative product (TPNH-X) when indicated, pH 7·4.

degradative products as contaminants, as shown by spectrophotometric studies. In fact, the ratio of absorbance at 260 mμ to that at 340 mμ varied from 3·4 to 4 or higher in many preparations, instead of being 2·4. The minimum absorbance between the peaks was also abnormal, with a displacement from 292 mμ to 305 mμ. These characteristics correspond to the presence of degradative products and they could be important, because it was shown that the degradative product

of TPNH obtained by incubating it in a boiling bath for a few minutes strongly inhibited glucose uptake, lactic acid production and phosphate disappearance (Fig. 9).

Nevertheless, one preparation of TPNH that had the spectrum corresponding to a very pure compound and that was further purified of possible metal contaminants by passing

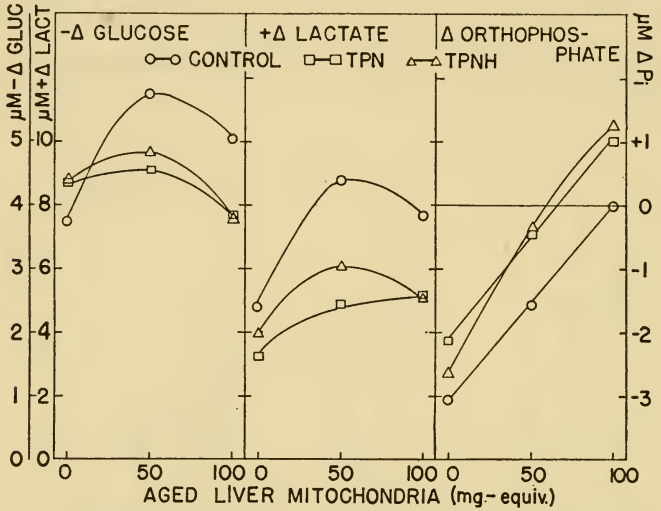

FIG. 10. Effect of TPN and TPNH on glycolysis. Influence of mitochondria. Each flask contained in a 1·5-ml. volume, 0·5 ml. brain supernatant, variable amounts of mitochondria, 7·5 μM glucose, 3 μM ATP, 3 μM ADP, 0·5 μM DPN, 0·1 μM cytochrome *c*, 6 μM-$MgCl_2$, 10 μM potassium phosphate pH 7·45, 35 μM Tris buffer pH 7·45. 3 μM TPN or TPNH when indicated. 0·15 M-KCl to maintain isotonicity.

it through a Dowex-50 column, exhibited an effect on glycolysis very similar to that of purified TPN (Fig. 10).

TPN-lactic dehydrogenase—To understand the mechanism of action of TPNH it was important to learn that brain supernatant has a TPN-dependent lactic dehydrogenase activity, similar to that found in rat liver (Navazio, Ernster and Ernster, 1957). This enzyme can bring about the oxidation of TPNH in the presence of pyruvate. It is not known if this is a separate enzyme from the DPN-lactic dehydrogenase. The

ratio of the two activities depends markedly on the pH, as is shown in Fig. 11. A similar influence of pH is found in the liver (Navazio, Ernster and Ernster, 1957) and in Novikoff tumour (Potter and Niemeyer, unpublished data). This fact and the finding that the ratio of activities is different from one tissue

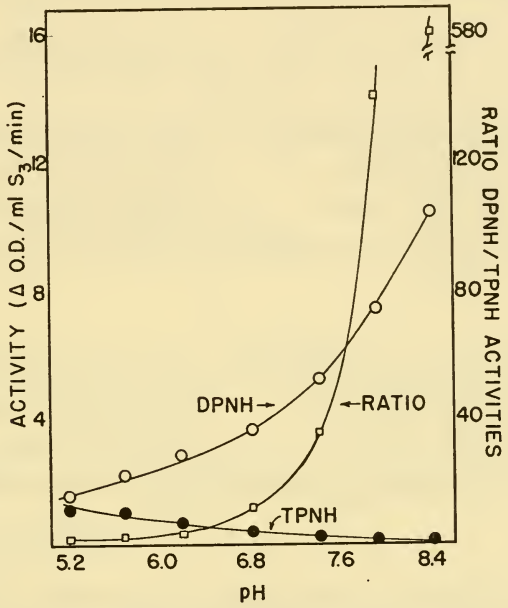

FIG. 11. Influence of pH on DPN- and TPN-linked dehydrogenase activities. The assay mixture contained brain supernatant 0·4, μM DPNH or TPNH, 5 μM pyruvate, Tris-maleate buffer of variable pH and water to complete 3·0 ml.

to another suggests that we are dealing with separate enzymes (cf. Rutter and Lardy, 1959). At the pH of 7·4 used in the glycolysis experiments, the activity of the TPN dehydrogenase would be enough to permit the oxidation of about 0·9 μM of TPNH by 0·5 ml of the brain supernatant in 30 minutes.

Addition of mitochondria—The addition of aged mitochondria increased the inhibitory effect of TPNH, or converted

a negligible to a significant inhibition. As occurred with TPN, when TPNH alone produced an increase in glucose consumption, the reverse effect was observed in the presence of mitochondria. Parallel to this effect of mitochondria was the conversion of TPNH into TPN, as is shown in Fig. 5. In fact, only when mitochondria were present could a net and

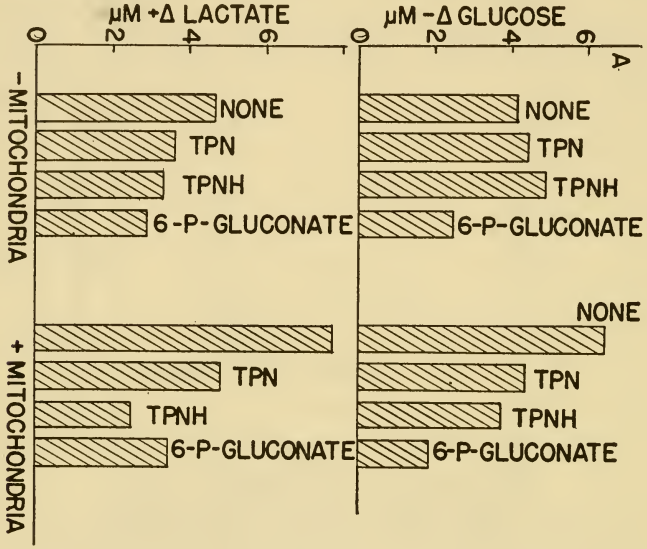

FIG. 12. Effect of 6-phosphogluconate on glycolysis. The composition of the medium was as in Fig. 2, but 0·1 μM cytochrome *c* was added. 100 mg.-equiv. of liver mitochondria were added as shown. 3 μM of triphosphopyridine nucleotides or 6-phosphogluconate were added when indicated. The bars represent the average of duplicate flasks for each experimental condition.

significant production of TPN from TPNH be demonstrated. This means that the effect of the TPN-lactic dehydrogenase was not enough to surpass the reductive activity of the dehydrogenases of the pentose cycle that operate in the system.

It must be pointed out that there was not a good correlation between the *amount* of the oxidized form of the coenzyme in the medium and the inhibitory effect on glycolysis, either with TPN or with TPNH added initially, in the presence

of mitochondria or without them (Fig. 5). In view of this lack of differentiation between TPN and TPNH effects, which is attributed to turnover of the coenzymes, and the fact that TPN did not inhibit directly the enzymes concerned in glycolysis, the possibility was considered that the triphosphopyridine nucleotides acted catalytically, producing indirectly some inhibitor.

Effect of 6-Phosphogluconate

There is evidence that 6-PG is able to inhibit competitively phosphohexoisomerase from different sources (Parr, 1956,

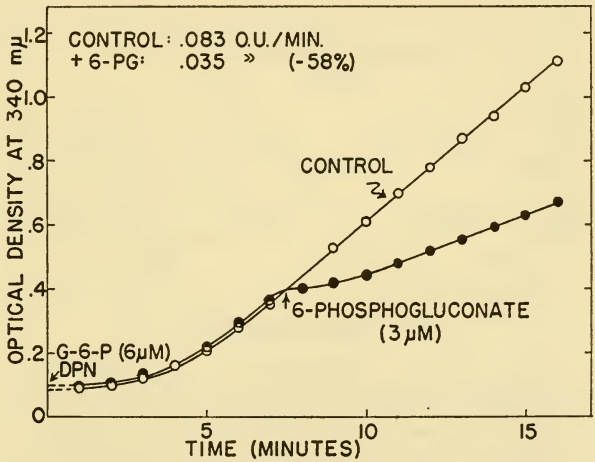

FIG. 13. Effect of 6-phosphogluconate on the reduction of DPN by G-6-P. The composition of the medium was similar to that used in Fig. 6, *right*, 6 μM G-6-P were used as initial substrate. 3 μM 6-PG when indicated.

1957). As TPN could act indirectly by producing 6-PG from G-6-P, the effect of 6-PG was investigated. It was shown that 6-PG strongly inhibited glucose uptake, lactic acid production and phosphate uptake, as shown in Fig. 12. Its effect is, in general, more pronounced than that of TPN or TPNH, and is also dependent on the pH of the system.

The effect of 6-PG was also shown in a system in which DPN was reduced with G-6-P as the initial substrate (Fig. 13). The

results were in agreement with an inhibition of phosphohexoisomerase.

If 6-PG plays any rôle in the mechanism of TPN action, one has to show that there is an accumulation of 6-PG under our experimental conditions. The prerequisite for such an accumulation is present in the system, since the assays indicated that the G-6-P dehydrogenase is more active than the 6-PG

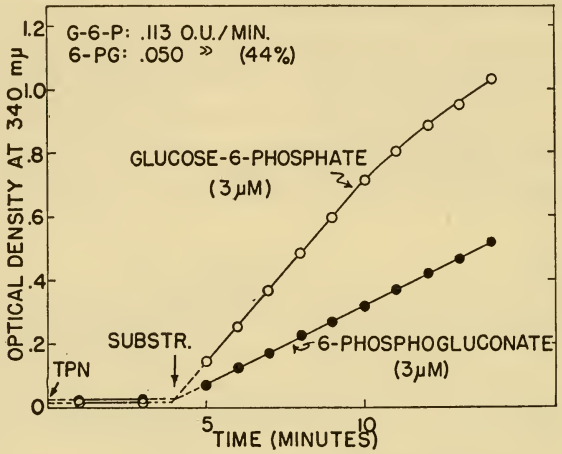

FIG. 14. Glucose-6-phosphate and 6 phosphogluconic dehydrogenase activities in brain supernatant fraction. The curve indicated by "Glucose-6-phosphate" represents the activity of glucose-6-phosphate dehydrogenase, corrected for the activity on 6-phosphogluconate, as described by Glock and McLean (1954).

dehydrogenase (Fig. 14). This relationship had also been shown by Glock and McLean (1954) and more recently by McNair Scott (1958). Preliminary experiments have shown that 6-PG accumulates in the presence of TPN, but not in its absence, with or without mitochondria.

Discussion

The experimental data support the conclusion that in the brain system the presence of TPN can change the rate of

glycolysis. The hypothesis is formulated that the TPN does not act directly, but indirectly by permitting the operation of the first reactions that initiate the pentose cycle. The relative rate of the reactions catalysed by the G-6-P dehydrogenase and by the 6-PG dehydrogenase would favour the accumulation of 6-PG in the system. This metabolite would inhibit competitively the phosphohexoisomerase, leading thus to an accumulation of G-6-P, which in turn would inhibit hexokinase, as has been shown to occur with hexokinases from animal sources (Weil-Malherbe and Bone, 1951; Crane and Sols,

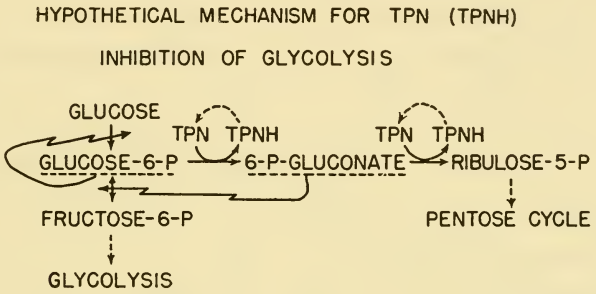

FIG. 15. Scheme to show the hypothetical mechanism of the inhibition of glycolysis by TPN.

1953). Fig. 15 shows schematically this feedback mechanism of control of glycolysis, indicating the sequence of events that would permit the inhibition of glucose consumption in the presence of TPN. To be effective in the formation of 6-PG, this mechanism requires the regeneration of TPN which can be accomplished by any oxidizing system that can couple with TPN. In addition to the intramitochondrial systems constituted by the TPN-cytochrome *c* reductase and by the transhydrogenase, others exist in the supernatant fraction that may perform this function. One of these is the TPN-lactic dehydrogenase, which can reduce some of the pyruvic acid formed during glycolysis. It is possible that other systems which utilize TPNH can act in the same way. Recent experimental data stress the importance of the conditions which

oxidize TPNH in the performance of the pentose cycle (Bonsignore, Pontremoli and Vergnano, 1957; Kinoshita, 1957; Cahill *et al.*, 1958; Wenner and Moliterno, 1958).

Some of the inter-relationships between several of the oxidation-reduction equilibria more directly concerned with carbohydrate metabolism and with the system used in our experiments are shown in Fig. 16. One must bear in mind, however, that there exist, both in aerobiosis and in anaerobiosis, several alternative pathways for hydrogen and for the

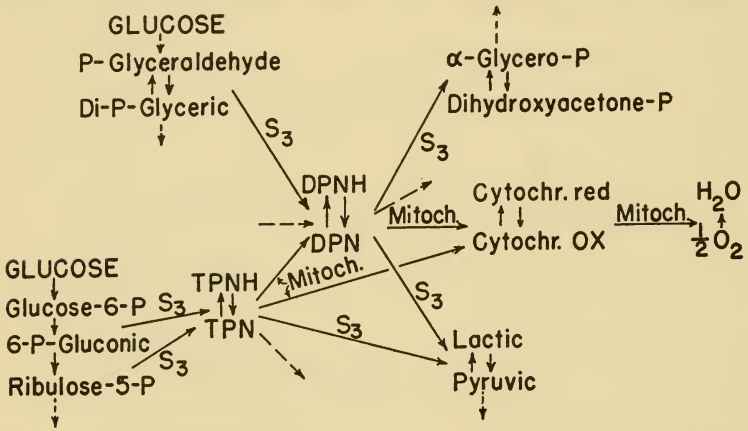

FIG. 16. Scheme to show some of the inter-relationships between different oxidation-reduction systems related directly to carbohydrate metabolism.

other metabolites of this scheme, but one has to realize that it would be impossible to try to integrate all these possibilities in a single figure.

The enhancement of glucose consumption observed in some experiments is understandable if one considers such a relationship of enzyme activities that would permit the shift of a fraction of G-6-P to the pentose cycle, enough to increase glucose uptake and to decrease lactic acid production, but still unable to produce enough inhibitor to act upon the isomerase. This shift of G-6-P metabolism from the glycolytic channel to the pentose cycle pathway was postulated by

Engelhardt (1939) to explain the Pasteur effect, but undoubtedly this mechanism alone does not explain the decreased glucose consumption, which we consider a *sine qua non* condition to define the Pasteur effect (Dixon, 1937).

The magnitude and quality of the effect of the TPN in a given preparation, or cell, would depend not only on the amount of coenzyme present, but as much on the relative efficiency of the enzymic reactions of the pentose cycle, in strict relationship with the systems that reoxidize TPNH. All these inter-related activities not only depend on the capacity of each particular enzyme, but also on other parameters, such as relative concentration of the substrates, presence of second substrates, and general conditions such as the pH of the reaction medium. The marked effect of pH in opposite directions upon DPN- and TPN-lactic dehydrogenase activities (Fig. 11) suggests the possibility that minor changes in pH may influence the overall equilibria, permitting the reduction of pyruvate either by DPNH or by TPNH, if the pH tends to be higher or lower than neutral, respectively.

The possibility that mechanisms involving TPN play a rôle in the Pasteur effect should be considered. We feel that enough experimental data exist to support the hypothesis that the Pasteur effect is obtained through a mechanism that involves a competition for inorganic phosphate of phosphate acceptors (Terner, 1954; Lynen, 1955; Gatt, Krimsky and Racker, 1956; Wu and Racker, 1957; Holzer, Witt and Freytag-Hilf, 1958). In fact, we have seen that this mechanism operates in cells to effect a balance between alternative pathways and to control their reactions at different levels in relation to the conditions of the surrounding medium. Even if the phosphate control is very important, it seems justifiable to look for other mechanisms that may operate under certain conditions.

Summary

The effect of the addition of triphosphopyridine nucleotides on the glycolytic activity of an enriched cell-free brain preparation was investigated. The oxidized and the reduced

form of the coenzyme decreased glycolysis, an effect that was enhanced markedly by the simultaneous addition of liver mitochondria, which can oxidize the TPNH.

The results are explained by an accumulation of 6-phosphogluconate, which inhibits phosphohexoisomerase. This block would permit an accumulation of glucose-6-phosphate which, in turn, would inhibit hexokinase. The relative activities of glucose-6-phosphate and 6-phosphogluconic dehydrogenases and the presence of a TPN-lactic dehydrogenase in the system contribute to the operation of this mechanism.

Note added in proof. The following summary of data relevant to our findings is taken from a recent publication (Neufach, S. A., and Melnikova, M. P. (1958). *Biochimia*, **23**, 440):

"It is assumed that rates of glycolysis may be determined by different factors under varying functional conditions of the cell and varying levels of glycolysis. The maximal level for rates of glycolysis in muscle *in situ* (about 1500 μM lactic acid per g. per hour) can be reproduced *in vitro* by means of a reconstructed system consisting of dialysed muscle extract ATP, DPN, Mg^{2+}, F-di-P, orthophosphate, nicotinamide, cysteine and glycogen. When the rate of glycolysis follows a linear course, the "slowest" enzyme of glycolysis may be identified directly by the acceleration of the process which occurs on introducing the purified enzyme. It is shown that none of the enzymes, aldolase, 3-phosphate dehydrogenase or lactic dehydrogenase, hitherto believed to to be the slowest enzymes of glycolysis, act as such in reality when their activity is displayed in a medium with sufficient coenzyme content. This is the condition prevailing in the body when muscle work is highly active. Depending on the value of 6-phosphofructokinase power, a 4- to 8-fold increase in the rate of glycogen disintegration follows, when purified PFK is introduced. Linear growth of the effect accompanies increasing increments of PFK. The rate of glucose disintegration may even increase to 10–14 times the original value when purified hexôkinase has been introduced. The same result has been obtained with a fresh, non-dialysed muscle extract.

It is suggested that, while the rate of glycolysis in skeletal muscle does depend upon PFK power, it is a function of hexokinase in nervous tissue, heart, erythrocytes and tumours."

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DISCUSSION

McIlwain: William of Ockham continues that entities should not be multiplied *more than is necessary.* Prof. Potter has introduced a new entity into the discussion: one composed partly of cerebral tissues and partly of preparations from the liver. It is interesting to speculate how this entity might differ from ordinary tissues. It is a peculiarity of the hexokinase in cerebral tissues that most of it is removed in the particulate matter during many types of extraction; so that your system might be

more limited than usual in the entry of glucose through all pathways. To what extent might this be responsible for some of the effects which you have seen?

Potter: It would be worth while to add more hexokinase to the experimental set-up and study the effect. Dr. Racker showed that when phosphate was pushed to a very high level, there was still a 19 per cent Pasteur effect. Without seeing the actual plot of the data one could not judge whether or not there is a certain amount of Pasteur effect which cannot be explained in the classical way.

Racker: I agree that we should keep in mind the possibility of multiple causes for the Pasteur effect. If this will be shown, we will be faced with the problem of evaluating quantitatively the relative contribution of the various mechanisms. My guess is that this will not be simpler than to quantitate the relative contribution of, e.g. the shunt pathway in glucose metabolism. Nevertheless, Prof. Potter, have you made attempts to determine the concentrations of glucose-6-phosphate and 6-phosphogluconate in your system and are they high enough to account for the observed inhibition? I ask, because Dr. Wu has determined the intracellular concentration of these two intermediates in ascites cells and they are too low to account for the Pasteur effect—unless we invoke the fashionable concept of compartmentation again.

Potter: I should like to relate why we got into this in the first place. Our original observations indicated that there was a lack of transhydrogenase and TPN cytochrome *c* reductase in certain tumour tissues. Then, also, the studies by Drs. Glock and McLean (1957, *Biochem. J.*, **65**, 413) indicated that TPN is very low. Therefore, I think that tumour tissues are precisely the tissues to which this mechanism does not apply; i.e. they may lack this system. That does not imply that those tissues cannot carry out this pathway. In most tissues this pathway may actually result in an increased disappearance of glucose because of a failure to pile up these intermediates which have a blocking effect.

Experiments carried out many years ago by Dr. LePage and myself (1946, *Amer. J. Physiol.*, **146**, 267) showed that glucose-6-phosphate exists in respectable amounts in tissues. Dr. Aisenberg (unpublished data) says that there appears to be an actual increase in glucose-6-phosphate in the presence of mitochondria.

Racker: The intracellular concentration of glucose-6-phosphate in ascites cells is well below the K_i value for product inhibition of hexokinase. We should also keep in mind that an increase in glucose-6-phosphate concentration *per se* cannot account for the Pasteur effect. To paraphrase a proverb, "You cannot eat your hexokinase and have it." If hexokinase makes enough glucose-6-phosphate so that it accumulates in respectable amounts, the removal of this compound rather than its formation must be limiting. I have also two mild objections to the proposal of inhibition of hexose isomerase. One is based on the very high cellular contents in hexose isomerase. The second is that we could not explain by this concept a Pasteur effect with fructose as substrate.

Lynen: I wonder whether the mechanism discussed can be used to

explain the effects of DNP and other uncoupling agents on the Pasteur effect.

Potter: I would emphasize that this may not play a rôle in the Pasteur effect. We have no data indicating a quantitative rôle. Dr. Racker's data on a 19 per cent Pasteur effect indicates that something else might be looked for. I think that in tissues other than those he used, the figure may be larger.

Greville: This is a slight change of front. Prof. Lipmann drew attention in the discussion after Dr. Racker's paper to the inhibition by DNP of assimilation and growth, in strictly anaerobic systems. DNP also has an effect in stimulating the lactic acid production by intact frog muscle, again under strictly anaerobic conditions, as was first shown by Ronzoni and Ehrenfest (1936, *J. biol. Chem.*, **115**, 749), and later confirmed in unpublished work by Dr. Dorothy Needham and myself. We found that mepacrine, another uncoupling agent, has the same effect. We assumed that this effect might be due to stimulation of the ATPase of the mitochondria in the cells, with consequent provision of ADP. But I gather that Prof. Lipmann and Prof. Lynen frown on this idea of ATPase working in the intact cell. Could somebody offer some other explanation of Ronzoni and Ehrenfest's effect?

Lipmann: I don't understand why it could not act on the ATPase. You still have to transport the glycolytic ATP into the mitochondria to get into this ATPase milieu and get out again. It must go in and out all the time.

Krebs: If Prof. Lipmann and others do not believe in ATPase, I take it that they assume that the various mechanisms which convert ATP into ADP and phosphate, are complex systems.

Racker: I believe Prof. Lipmann wants to go one step further. He suggests that the hydrolysis of ATP by ATPase is not functional inside the cells and DNP does not activate it *in vivo* as it does *in vitro*.

Lipmann: I had that in mind, but I do not know if I can stick to it!

Greville: We confirmed that DNP stimulates the ATPase activity of mitochondria isolated from frog muscle.

Magasanik: What is the general effect of DNP on growth? In *Aerobacter aerogenes* we have been unable to inhibit the growth of the organism on glucose with DNP. We put DNP in to saturation, and still the organism grew. Yet, in resting cells a very definite DNP effect can be shown—there is an increased oxygen uptake and no assimilation of carbohydrate.

Lehninger: Does anyone know whether DNP inhibits assimilation in a strict anaerobe?

Slater: Prof. Lipmann has drawn attention to the important point that DNP has an effect on anaerobic metabolism. This interested me also some time ago. I wonder whether some of these effects could be explained by a DNP-stimulated hydrolysis of what we now call X$\sim$Y. In other words, you do not believe in the ATPase reaction in the cell, but a part of it, the reaction of ATP with X and I to form X$\sim$I, might be involved in the utilization of ATP formed by glycolysis or fermentation.

PHOSPHATE TURNOVER AND PASTEUR EFFECT

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In 1941, Lynen and Johnson developed independently a theory of the Pasteur effect. This effect is the inhibition of fermentation by respiration, and could be explained in terms of appropriation of intracellular inorganic phosphate by the respiratory system. The theory was based on Harden and Young's (1905) fundamental discovery that sugar fermentation depends on the presence of free phosphate. Later, Warburg and Christian (1939) found the chemical explanation for this phenomenon when they studied the mode of action of crystalline triose phosphate dehydrogenase. They performed kinetic measurements of the dehydrogenation reaction, which is the keystone of energy production in fermentation, and found that it requires inorganic phosphate, the concentration of which can thus control the reaction rate. Following the discovery that respiration, also, is dependent on inorganic phosphate (Engelhardt, 1932; Kalckar, 1937; Belitzer, 1939), the ground was prepared for our theory of the Pasteur effect: under aerobic conditions respiration competes with fermentation for the inorganic phosphate in the cell.

This metabolic regulation of the living cell is controlled by the "phosphate cycle". By this term the compensation of adenosine triphosphate (ATP) synthesis by the cleavage reactions yielding inorganic phosphate may be described. The ATP synthesized in exergonic catabolism is used for the great variety of energy-requiring reactions connected with the process of life. The two parts of the cycle, phosphorylation and dephosphorylation, have to be equilibrated against each

other. Without this cycle inorganic phosphate, adenosine diphosphate (ADP) and ATP could not fulfil their important catalytic functions in the cell. If, in the living cell, this equilibrium were not maintained, all metabolism would cease within a short time, due to lack of ATP if phosphorylation proceeded too slowly as compared with dephosphorylation, or due to lack of inorganic phosphate and ADP in the case of too rapid phosphorylation. Thus dephosphorylation, which depends directly on the energy requirement of the cell, may regulate the catabolic processes and correlate them with the energy

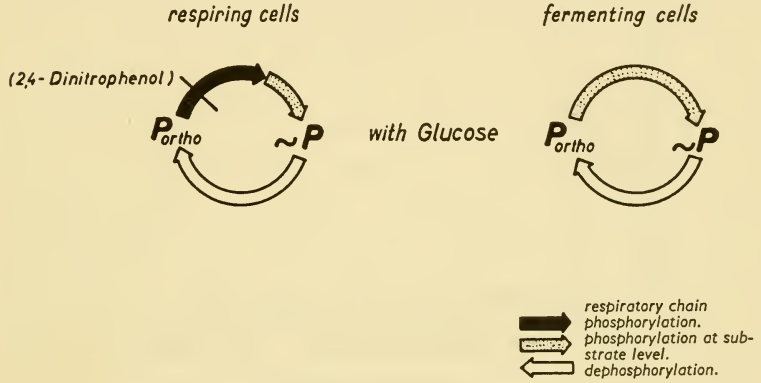

FIG. 1. "Phosphate cycle" in living cells.

requirement (cf. Lardy, 1955). The inhibition of fermentation by oxygen would, therefore, depend on the following facts: under aerobic conditions, respiration and fermentation must share the dephosphorylation capacity which is completely available for fermentation under anaerobic conditions. These facts are represented schematically in Fig. 1, assuming aerobic and anaerobic dephosphorylation to be equal. The latter was confirmed by experiments with yeast cells (Lynen and Koenigsberger, 1951). It seemed that the aerobic inhibition of fermentation was due to the decreased amount of inorganic phosphate available for the triose phosphate dehydrogenation; evidence in support of this was obtained by phosphate analysis of respiring and fermenting yeast cells

(Lynen, 1941). It was found that the concentration of inorganic phosphate under oxygen was lower than under nitrogen. If yeast cells are suspended in glucose solution and shaken alternatively under oxygen and nitrogen, the free phosphate oscillates between the lower aerobic and the higher anaerobic level (Fig. 2). However, the differences observed do not seem to be large enough to explain the considerable slowing down

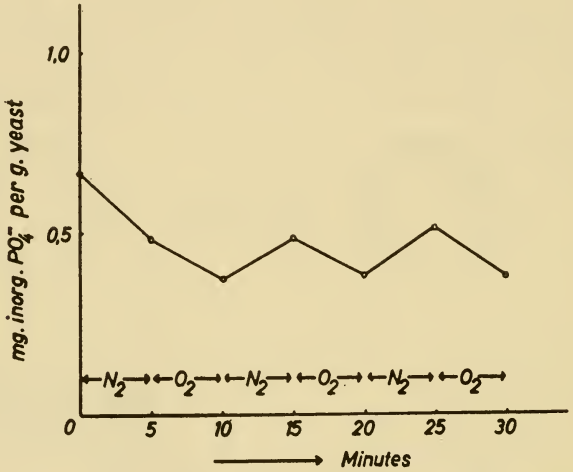

FIG. 2. The orthophosphate content of baker's yeast, aerobically and anaerobically.
Yeast, 10 per cent; glucose, 6 per cent; under oxygen and nitrogen alternatively; temp. 30°.

of triose phosphate dehydrogenase caused by respiration. In an attempt to overcome the difficulty, we considered the possibility of some of the inorganic phosphate in the cell, as determined by the molybdenum blue method, being present not as free phosphate, but as having existed as some labile phosphorus compound, e.g. acyl phosphate, which had become hydrolysed during assay (Lynen, 1941; Kamen and Spiegelman, 1948). More recent observations and the negative result of attempts to find noticeable amounts of acyl phosphate in respiring or fermenting yeast cells (Holzer, 1949) made it

necessary to modify this hypothesis. As is well known today, membranes divide the cell into numerous compartments with different enzyme patterns. The transport of materials between these various compartments is more or less limited. The membranes separating cytoplasm from mitochondria are apparently of particular importance for the Pasteur effect, as shown below. The phenomenon of adsorption on certain cell structures may also lead to an unequal distribution of material. Finally, mention should be made of the many specific enzyme-substrate and enzyme-coenzyme complexes. The situation is such a complicated one that an exact analysis of this pattern is not to be expected at present.

That inorganic phosphate takes part in this uneven mosaic-like distribution inside the cell is supported by data obtained by Trevelyan, Mann and Harrison (1954). The fact must be borne in mind that the metabolic degradation of sugar in yeast, aerobically as well as anaerobically, is coupled to the synthesis of glycogen-like polysaccharides. In fact, a considerable part of the sugar metabolized is assimilated in this process. The phosphorylase-catalysed glycogen synthesis from glucose-1-phosphate has been found to occur in yeast extracts (Trevelyan, Mann and Harrison, 1954; Whelan, 1955). However, this process can proceed only if the ratio of inorganic phosphate to glucose-1-phosphate in the cell is lower than the equilibrium constant of the phosphorylase reaction. Otherwise, glycogen will be degraded by phosphorylation. When the ratio of inorganic phosphate to glucose-1-phosphate was determined in yeast cells assimilating sugar, Trevelyan, Mann and Harrison (1954) surprisingly found a value much higher than the equilibrium constant. Their findings are confirmed by some data obtained by Netter and Schuegraf (1958, unpublished) in our laboratory (Table I). The discrepancy between experiment and theory can only be eliminated by assuming an unequal distribution of these phosphates in the inner compartments of the cell. One possibility would be that the volume available for the hexose phosphates would be substantially smaller than that available for

Table I

ORTHOPHOSPHATE AND HEXOSE PHOSPHATE CONTENT OF BAKER'S YEAST, RESPIRING IN GLUCOSE SOLUTION

Orthophosphate	*Glucose-6-phosphate*	*Glucose-1-phosphate (calc.)**	*Orthophosphate/ glucose-1-phosphate†*
3·90	1·44	0·073	53·5
3·30	1·48	0·075	43·0
1·65	0·88	0·044	37·5
1·60	1·00	0·050	32·0
3·75	2·04	0·103	36·4
1·50	0·72	0·036	41·5 (15° C)

* phosphoglucomutase: $K = \frac{\text{glucose-6-phosphate}}{\text{glucose-1-phosphate}} = 19{\cdot}8$ (Trevelyan, Mann and Harrison, 1954).

† phosphorylase: $K = \frac{\text{orthophosphate}}{\text{glucose-1-phosphate}} = 6{\cdot}3$ (Trevelyan, Mann and Harrison, 1954).

inorganic phosphate. Another explanation could be based on the different orthophosphate levels in the cell compartments, due to the unequal enzyme distribution. The latter idea is supported by experiments where yeast cells were plasmolysed by freezing in liquid air, the thawed material then separated by immediate centrifugation into a "supernatant" containing the elements of the cytoplasm, and a "residue" with the structure-bound elements of the cell (Holzer and Lynen, 1950). Actively respiring yeast cells had a considerably higher percentage of their total inorganic phosphate in the "residue" than had fermenting or "starved" cells. We may assume that this unequal distribution is based on the accumulation of inorganic phosphate in the particulate areas of the cell, e.g. the mitochondria.

From these experiments we may conclude that any interpretation of phosphate determinations in terms of actual phosphate concentration is only of limited value. However, some information about the situation at the sites of active metabolism may be obtained by kinetic measurements. They can indicate in an indirect way what the turnover of inorganic and organic phosphates inside the cell is like (Lynen and Koenigsberger, 1951; Lynen *et al.*, 1958).

Before we go into the details of our findings, the principal features of the Pasteur effect must be described further. As

yet, only the inhibition of glucose degradation via triose phosphate has been considered. The Pasteur effect, however, also becomes evident in the aerobic decrease of sugar uptake by the cell. This can be demonstrated in a simple way by suspending equal amounts of yeast cells in sugar solutions. One sample is kept aerobically, the other anaerobically, and both are assayed for the disappearance of glucose from the medium. It is found that the glucose decreases more rapidly with fermenting yeast cells than it does with respiring cells (cf. Table II).

Table II

BALANCE OF GLUCOSE UPTAKE, AEROBICALLY AND ANAEROBICALLY

$T = 25°$	Q Glucose		
	Anaerobically	*Aerobically*	
2 : 4-*Dinitrophenol*	—	—	4×10^{-4} M
Uptake of glucose	3·085	1·950	3·020
Degradation by fermentation	1·945	0·343	1·387
Degradation by respiration	—	0·356	0·556
Total glucose degradation	1·945	0·699	1·943
Assimilation	1·145	1·251	1·077

Our experiments rule out the possibility that oxygen changes the cell-wall permeability. If an effect of that kind existed, we should expect the inter-relationship of sugar uptake and sugar concentration in the medium to be different under aerobic and anaerobic conditions. Hartmann (1958, unpublished), in our laboratory, has studied this problem systematically. Of course, his measurements had to be restricted to low sugar levels where no Pasteur effect occurs. The technique used was as follows: a mechanically driven syringe delivers sugar solution at a constant rate into a large volume of yeast suspension. At certain intervals the sugar concentration of the solution is measured. Fig. 3 shows the technical set-up. It was found that a constant sugar concentration is maintained, which means that the addition of sugar from the syringe and the uptake of the yeast cells correspond to each other. By varying the sugar concentration of the

solution to be delivered from the syringe we can experimentally change the rate of sugar uptake. Thus, various constant sugar levels in the cell suspension can be maintained.

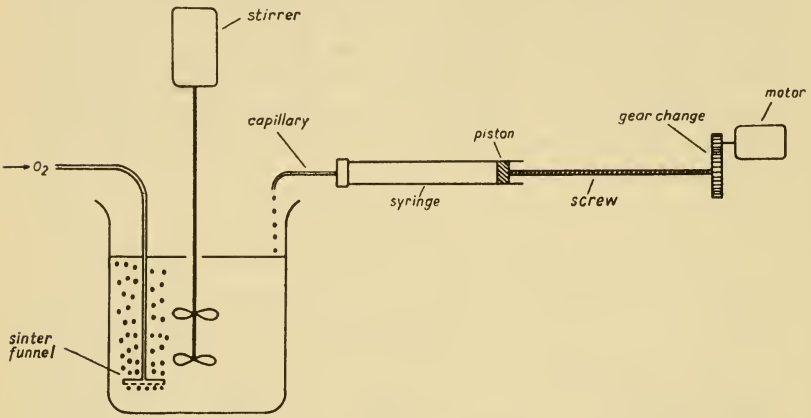

FIG. 3. Experimental set-up for measurement of sugar uptake by yeast cells.

The results of Hartmann's measurements are presented in Fig. 4. In the present discussion we are mainly interested in

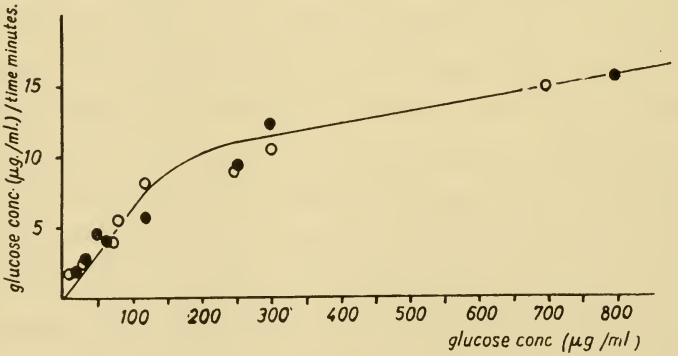

FIG. 4. Glucose uptake by baker's yeast aerobically and anaerobically.
Yeast, 1 per cent in buffer, pH 5·4; temp. 20°. Abscissa: glucose concentration in the solution. Ordinate: rate of glucose addition to the yeast suspension, ○ = aerobic ● = anaerobic.

the fact that it makes no difference whether the yeast cells are kept aerobically or anaerobically. Obviously the permeability of the cell membrane, which could control the sugar uptake, is independent of the gas phase.

The peculiar form of the curve may be expressed by two

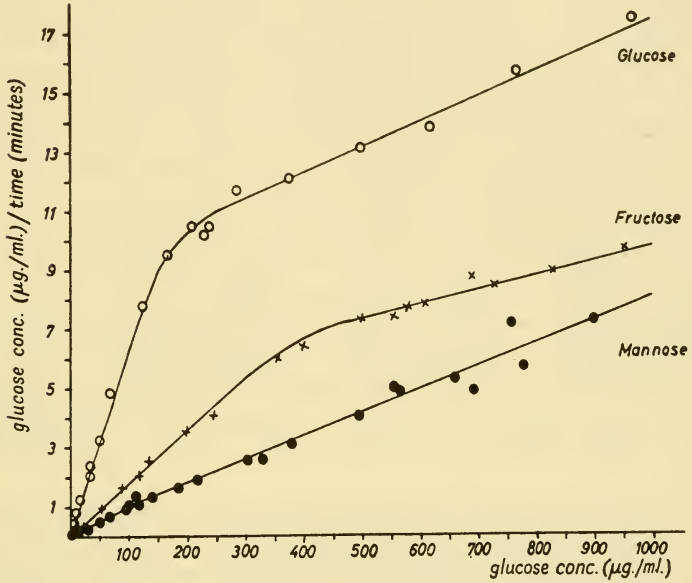

FIG. 5. Comparison of uptake of glucose, fructose and mannose; aerobic.
Experimental conditions as for Fig. 4.

straight lines with different slopes (Figs. 4, 5). This suggests that two processes overlap when glucose enters the yeast cell. One process is probably an "active" transport and dominates in the range of small substrate concentrations from zero up to 0·001 M glucose. Under the conditions of the experiments this mechanism can transport up to 5 µmoles per minute per g. of wet yeast cells, which amounts to nearly half the saturation value. A second and much slower process is superimposed on the first one and can be assumed to be the passive sugar

diffusion through the cell wall. The active mechanism seems to be rather specific for glucose. To a lesser extent it is involved in the uptake of fructose (Fig. 5), whereas it cannot be observed in experiments with mannose. Yet, according to Hartmann's experiments, in the range of full substrate saturation all three sugars are metabolized at equal rates.

Since these experiments had eliminated permeability changes as the cause of the decreased aerobic sugar uptake, other mechanisms had to be considered. Glucose metabolism starts with a phosphorylation reaction; therefore, the hexokinase reaction was examined next. If this enzymic process were to proceed more slowly aerobically than anaerobically, the decreased glucose uptake under oxygen would be explained. Experiments carried out with dinitrophenol (DNP) poisoning suggest that the decreased uptake must be related in some way to oxidative phosphorylation. As shown in Table II, in the presence of DNP aerobic glucose consumption increases to the value observed under anaerobic conditions. It should be mentioned that glucose degradation undergoes the same changes, as has long been known (Field, Martin and Field, 1935; Krahl and Clowes, 1935; Pickett and Clifton, 1941).

In order to explain the effect of oxidative phosphorylation on the glucose uptake, we suggested (Lynen and Koenigsberger, 1951) that ATP may be removed from the hexokinase and transferred to other sites in the cell, when respiration starts. This assumption was based on the fact that the respiratory enzymes are localized in the mitochondria, the fermentation enzymes, however, in the cytoplasm (Schneider, 1955; Nossal, 1954; Linnane and Still, 1955; Holzer and Goedde, 1957). The decreased glucose uptake under oxygen, therefore, would be a consequence of a transport phenomenon inside the cell. The mitochondrial membrane seems to act as a barrier for the ATP on its way from the mitochondria to hexokinase. In the case of fermenting cells the ATP generated in the cytoplasm is readily available for the hexokinase, whereas in the respiring cell ATP has to shuttle back and forth

between mitochondria and cytoplasm. Racker (1956) drew an analogous conclusion from his experiments on ascites tumour cells; so did Chance and Hess (1956) from their spectrophotometric experiments. The results obtained by the latter authors are conclusive proof for the slow phosphorylation of glucose by intramitochondrial ATP. The slow diffusion of

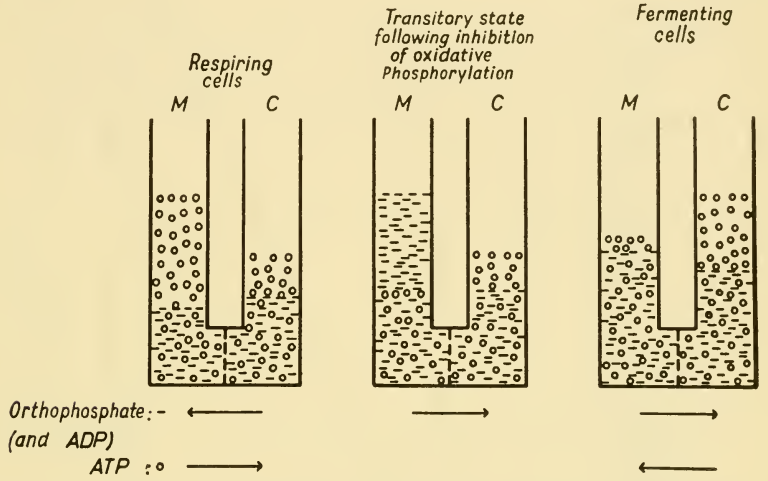

FIG. 6. Schematic representation of the intracellular distribution of inorganic phosphate, ADP and ATP in respiring and fermenting cells. M = mitochondria; C = cytoplasm. Vertical broken line represents barrier between mitochondrial and cytoplasmic compartments. Arrows indicate the directions of transport of inorganic phosphate and ADP (—) or of ATP (○) in the various states.

ATP from the mitochondria to hexokinase leads to depletion of ATP at the site of this enzyme. Simultaneously the ATP content of the mitochondrial areas increases. The intracellular ADP distribution may be expected to have the complementary pattern. This could explain why sugar phosphorylation can occur at different rates even if the total amounts of ATP and ADP remain unchanged. The stimulation of sugar uptake by DNP fits well into this picture. After the oxidative phosphorylation has been blocked the level of inorganic phosphate rises and initiates an increased fermenta-

tion; ATP is synthesized in the cytoplasm again, and consequently the rate of sugar phosphorylation increases to the anaerobic value. The pattern of the adenine nucleotides and

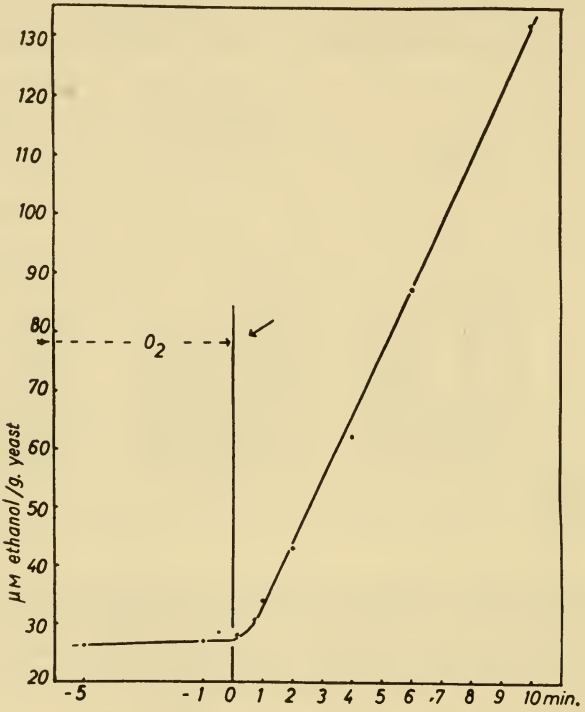

FIG. 7. Initiation of fermentation by cyanide. Yeast, 10 per cent; glucose, 2 per cent; in 0·02 M citrate buffer, pH 5·4; temp. 15°; oxygenated; 0·001 M-KCN added at arrow. Rate of fermentation was determined by the accumulation of ethanol.

the inorganic phosphate in respiring and fermenting cells is depicted in Fig. 6.

Experimental support for this theory was provided by a series of experiments carried out by Netter and Schuegraf (1958, unpublished). The changes of inorganic phosphate, ADP, ATP and hexose phosphates were followed when

energy production switched from respiration to fermentation and *vice versa*. If it is true that inorganic phosphate governs metabolism, this should be evident from the time sequence of events. The respiration of a vigorously aerated suspension of yeast cells in sugar solution is poisoned by 0·001 M cyanide;

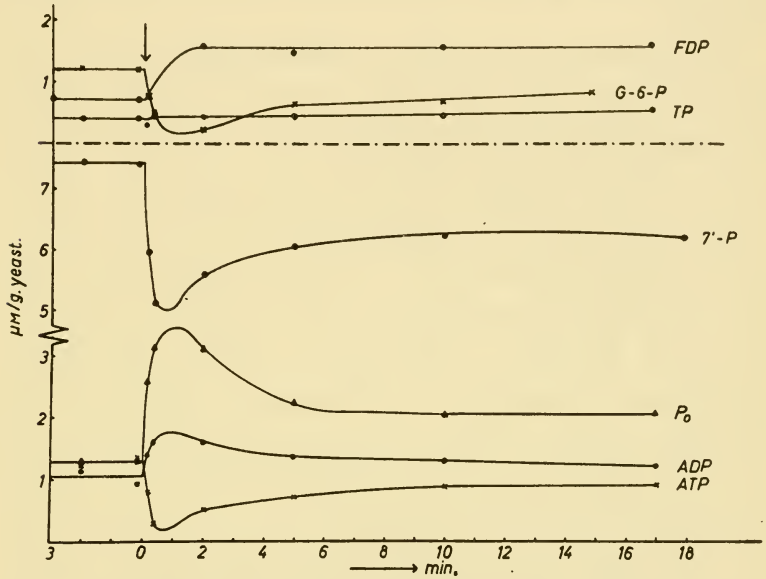

FIG. 8. Changes in inorganic and organic phosphates of baker's yeast, following the transition from repiration to fermentation.
Yeast, 10 per cent; glucose, 2 per cent; in 0·02 M citrate buffer, pH 5·4; temp. 15°; oxygenated; 0·001 M-KCN added at arrow. P_0 = orthophosphate; 7'-P = labile phosphate, split by 7 minutes' hydrolysis in N-HCl at 100°; G-6-P = glucose-6-phosphate; FDP = fructose diphosphate; TP = triose phosphate.

fermentation starts immediately, and under our experimental conditions reaches its steady state within 2 minutes (Fig. 7). The most striking event following the inhibition of respiration is a rapid increase in the inorganic phosphate of the cell, derived from the "labile phosphate" fraction (Fig. 8). At the same time ATP decreases and ADP increases correspondingly. All these changes are easily understood as the result of

the blocked oxidative phosphorylation. In the first period, therefore, dephosphorylation exceeds phosphorylation. For our discussion it is important that glucose-6-phosphate changes in parallel with ATP, and drops rapidly after respiration has been blocked. This leaves no doubt that, in yeast, mitochondrial ATP can be used for the initial phosphorylation of sugar.

The behaviour of fructose-1 : 6-diphosphate is quite unexpected: no change takes place immediately after cyanide poisoning; after 10 seconds, the fructose—1 : 6-diphosphate begins to rise until after about 2 minutes it reaches the higher level which corresponds to the anaerobic steady-state value. There are three possible explanations for this experimental result, which has been confirmed many times. Firstly, phosphofructokinase might have a much higher affinity for ATP than hexokinase. Lowering the ATP level, then, could have only little influence on the rate of fructose diphosphate formation. This theory was ruled out when Bryant (1958, unpublished), working in our laboratory, determined the Michaelis constant of the yeast enzyme, and found it to be close to the Michaelis constant of yeast hexokinase. Secondly, the rate of the ATP-dependent formation of fructose diphosphate might decrease simultaneously with the ATP level. At the same time, however, this decreased formation could be compensated by the diminished fructose diphosphate degradation. For instance, lack of DPN, following cyanide poisoning of respiration, would inhibit its further oxidation via triose phosphate. In fact, Chance—by means of his spectrophotometric method (Chance and Hess, 1956)—found that in the initial period after cyanide addition DPN is almost completely reduced. It takes 2–3 minutes before the stationary DPN/DPNH ratio of fermentation is reached (Fig. 9). However, it is possible that this fast DPNH formation may actually be due to triose phosphate dehydrogenation which, by reason of the cell being flooded with inorganic phosphate, is out of control for a short while. Experiments with DNP show that depletion of oxidized DPN cannot

account for the behaviour of fructose diphosphate (Fig. 10). DNP which, contrary to the action of cyanide, induces fermentation without inhibiting respiration, initiates the same changes in inorganic phosphate and hexose phosphates. In this experiment, too, glucose-6-phosphate drops rapidly whereas

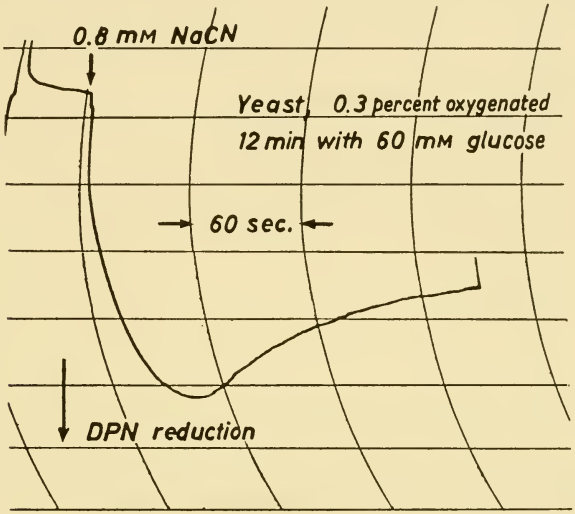

FIG. 9. DPNH formation following the blocking of respiration by cyanide. The ordinate values refer to changes in concentration of compounds specified.

fructose diphosphate is first maintained at a constant level and then rises.

These experiments suggest that oxidative phosphorylation is not related to the rate of fructose diphosphate formation. This would mean that mitochondrial ATP can phosphorylate glucose by hexokinase, but has little or no action on the further phosphorylation of hexose phosphate to form fructose diphosphate. Probably the two enzymes are located at different sites in the cell. If it is true that phosphofructokinase reacts only with cytoplasmic ATP, it is obvious that the fructose diphosphate level will correspond somehow to the

rate of fermentation. Thus, if the Pasteur effect is eliminated by cyanide or an uncoupling agent, the induced fermentation leads after a short transitional period to the increase in fructose diphosphate which was observed experimentally (Figs. 8, 10). Simultaneously ATP, the polyphosphates and glucose-6-phosphate increase, due to the gradual substitution of

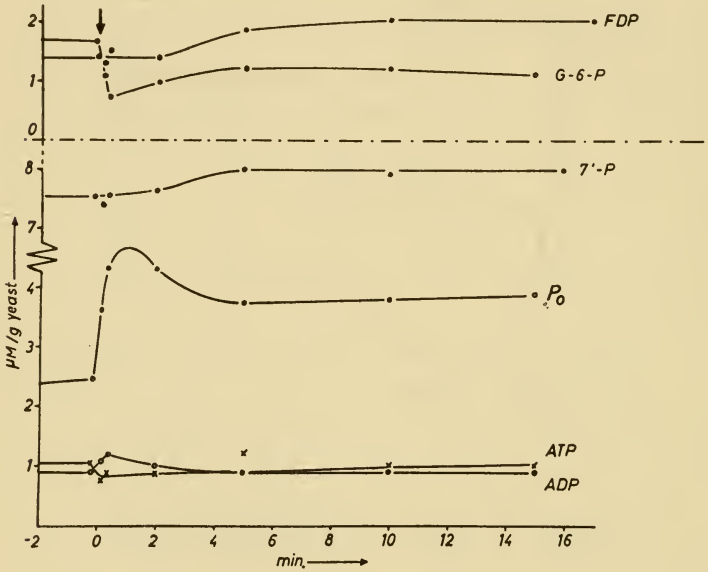

FIG. 10. Changes in the inorganic and organic phosphates of baker's yeast, following the addition of 2 : 4-dinitrophenol.
Yeast, 10 per cent; glucose, 2 per cent; in 0·02 M citrate buffer, pH 5·4; temp. 15°; oxygenated; 4×10^{-4} M dinitrophenol added at arrow. The ordinate values refer to changes in concentration of compounds specified.

energy supply by fermentation. The inorganic phosphate decreases again and is maintained at a higher level than before cyanide addition, as is required by the theory.

The changes observed on switching from fermentation to respiration also fit this picture. It is remarkable that ATP and ADP are not affected by aeration, as shown experimentally (Fig. 11). This is difficult to reconcile with the theory,

proposed by Chance and Williams (1956), that ADP controls the fermentation rate. The decrease of fructose diphosphate to the aerobic level shows a lag period and seems to reflect the low cytoplasmic orthophosphate concentration remaining available for fermentation under the aerobic conditions.

The behaviour of glucose-6-phosphate needs further explanation. In the experiments cited above, it was also found

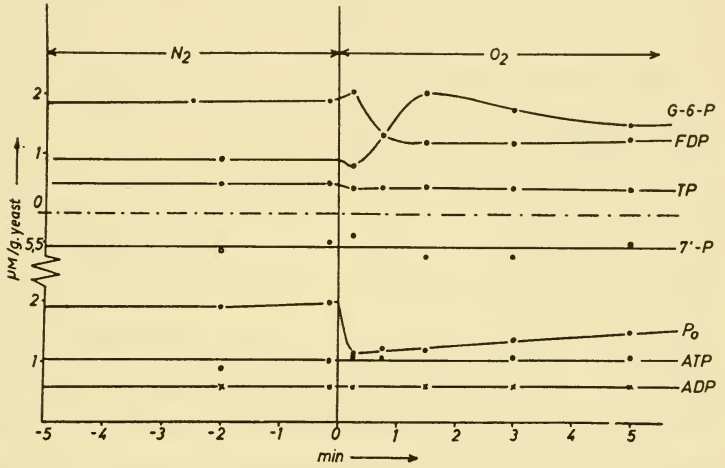

FIG. 11. Changes in the inorganic and organic phosphates of baker's yeast accompanying the transition from fermentation to respiration. Yeast, 10 per cent; glucose, 2 per cent; in 0·02 M citrate buffer, pH 5·4; temp. 15°; under nitrogen; oxygenated at zero time by bubbling oxygen through the yeast suspension. The ordinate values refer to changes in concentration of compounds specified.

that the steady-state level of glucose-6-phosphate is higher under aerobic than under anaerobic conditions. Two different explanations fit such an experimental finding: (i) a faster formation of the intermediate in the reaction chain, or (ii) a slower consumption. The first mechanism is not involved here. As pointed out already, one of the main features of the Pasteur effect is the lowered phosphorylation of glucose in respiring cells. Thus, the only remaining possibility is that the further transformation of glucose phosphate is diminished. As

it is unlikely that the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate would be affected by oxygen, the phosphofructokinase reaction must be the step concerned. Engelhardt and Sakov (1943) considered the aerobic inhibition of phosphofructokinase to be responsible for the Pasteur effect. They assumed an oxidative inactivation of the enzyme. This assumption, however, is not in agreement with the finding that DNP and other uncoupling agents, even in the presence of oxygen, suspend the enzyme inhibition. Aisenberg and Potter (1957) also observed an aerobic decrease in fructose diphosphate when they studied the Pasteur effect in artificial systems consisting of rat liver mitochondria and soluble fractions from rat brain and tumour tissue. They proposed that a high-energy intermediate of oxidative phosphorylation linking the respiratory chain and ATP is responsible for the inhibition of phosphofructokinase. While further experimental evidence for this hypothesis is lacking, we prefer a different explanation for the high glucose-6-phosphate level, concomitant with the decrease in fructose diphosphate in our experiments. It is based on our assumption, made before, that only cytoplasmic ATP originating from fermentation can be used for hexose phosphate phosphorylation. If this is correct, the decrease in fructose diphosphate would be due to the depletion of ATP in the cytoplasm and this would be the direct consequence of the respiratory inhibition of fermentation.

In summary, the results of our experiments with yeast cells indicate that the Pasteur effect is due to oxidative phosphorylation and enzyme localization in the different cell compartments. The phosphorylation reactions going parallel with the respiratory process exhaust the inorganic phosphate pool available for the dehydrogenation of glyceraldehyde phosphate, thereby inhibiting the fermentative glucose degradation. The reduced glucose uptake of respiring cells compared with that of fermenting cells is interpreted as an impairment of the hexokinase reaction due to lack of ATP at the site of glucose phosphorylation. The ATP formed during respiration

is accumulated in the mitochondria and so is not readily available for extramitochondrial glucose phosphorylation.

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DISCUSSION

Potter: My question is based on the assumption that ATP is pulled out of the cytoplasm into the mitochondria. It is a question of whether one can generalize from the yeast cell to other types of cell. Even in the

yeast cell there are many functions, e.g. protein synthesis, taking place in the cytoplasm. In the case of muscle it is possible to do a higher level of work under aerobic than under anaerobic conditions, at a sustained rate. Is it not necessary to make an *ad hoc* theory to explain how the ATP, which is not in the cytoplasm as far as the phosphofructokinase is concerned, *is* in the cytoplasm as far as certain functions are concerned? I agree that the endoplasmic reticulum offers an attractive way out of the dilemma, but I insist that it is an *ad hoc* hypothesis.

Lynen: There is the possibility that the Michaelis constants for the various processes differ. If the affinity of ATP for the protein-synthesizing enzyme were greater than for the phosphofructokinase, protein synthesis could occur at normal rates whereas the phosphorylation of phosphofructose would be retarded.

Coxon: A point relevant to the possible interaction of ATP in the mitochondria on the one hand, and the cytoplasm on the other, and one which draws on some electrophysiological evidence of a different kind from what we have been discussing up to now, is the observation that one can reduce the sodium efflux in the invertebrate nerve by soaking it in cyanide (Caldwell, P. C., and Keynes, R. D. (1957). *J. Physiol.*, **137**, 12) or in DNP (Keynes, R. D. (1959). *Proc. IV int. Congr. Biochem.*, in press); this is accompanied by lowering of the total ATP. On the other hand, after cyanide poisoning, one can restore the efflux by introducing ATP through an intracellular micro-injector; however, they did find that the restoration of the flux for a given concentration of ATP was less when that had been introduced from the outside, which suggested (Hodgkin, A. L. (1957). *Proc. roy. Soc. B*, **148**, 1) that they had not got it in quite the right place to give the maximum effect.

Holton: If a reversible transfer of ATP, ADP and inorganic phosphate in and out of the mitochondria really occurs, as has been postulated in connexion with the mechanism of the Pasteur effect, the movement of these substances might be accompanied by changes in mitochondrial shape or volume, and might thus be amenable to detection and study by optical methods. Dr. Beechey and I have recently made some observations on heart sarcosomes which show that a rapidly reversible swelling and shrinking phenomenon does indeed occur in these particles and that its properties are those to be expected from a transfer process linked closely with oxidative phosphorylation.

Relationships between transfer of material, morphological changes and active metabolism in mitochondria and sarcosomes have been reported by a number of workers (e.g. Bartley, H., and Davies, R. E. (1952). *Biochem. J.*, **52**, 20; MacFarlane, M. G., and Spencer, A. G. (1953). *Biochem. J.*, **54**, 569; Chappell, J. B., and Perry, S. V. (1954). *Nature* (*Lond.*), **173**, 1094; Beyer, R. E., Ernster, L., Löw, H., and Beyer, T. (1955). *Exp. Cell Res.*, **8**, 586), while recently it has been shown (Lehninger, A. L., and Ray, B. L. (1957). *Biochim. biophys. Acta*, **26**, 643) that a close link exists between the oxidation state of the respiratory chain of liver mitochondria and the occurrence of phosphate-induced swelling. In our experiments we have studied a particular

swelling process which occurs in heart sarcosomes under carefully defined conditions (Beechey, R. B., and Holton, F. A. (1959). *Biochem. J.*, in press). We took care that the extinction changes which we measured were due to morphological changes and not to changes in the oxidation states of respiratory pigments by following extinction changes at three wavelengths simultaneously. The swelling process which we observed may be related to some of the effects previously reported by other workers, but it was distinguishable from many of them by the following features. (1) The swelling was observed only under aerobic conditions and was rapidly and completely reversed when conditions became anaerobic. (2) Reversible swelling occurred in sarcosomes exhibiting respiratory control as defined earlier in this meeting by Prof. Chance (i.e. their respiration was accelerated by ADP and it became slower again when the added ADP was exhausted), but reversible swelling did not occur under a variety of conditions where respiratory control was shown to be absent.

Our observations included the changes of pigment extinction due to reduction of the respiratory chain as oxygen became exhausted, and we were able to show that anaerobic swelling gave place to anaerobic shrinking within a few seconds of the beginning of the shift of the oxidation state of the chain towards its anaerobic level. We also found that gradual loss of respiratory control during ageing was accompanied by a gradual disappearance of the reversible swelling phenomenon, and that a low concentration of DNP (4·6 μM) had the same effect.

We cannot yet say for sure that the aerobic swelling is due to the entry of inorganic phosphate and ADP into the sarcosome, or that the anaerobic shrinkage is due to their extrusion, but it seems possible that this is so, and that the observed link between reversible swelling and respiratory control represents part of the mechanism which controls the distribution of phosphate and adenine nucleotides within the cell.

Holzer: Prof. Lynen, are hexokinase and phosphofructokinase localized in the particles which can be obtained from yeast cells with your method?

Lynen: Dr. Bryant (unpublished experiments) investigated the distribution of both kinases, using yeast cells disintegrated in the Nossal shaker. When he determined the ratio of hexokinase to phosphofructokinase in the particulate fraction and in the supernatant, he found no difference. I may add that the activity of both enzymes in the isolated particles was extremely low.

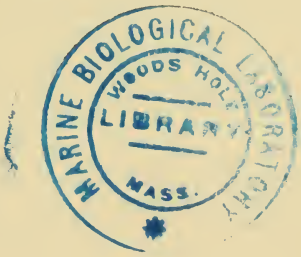
Hess: The Pasteur effect might be differentiated from ours and Crabtree's effect as follows. In the Pasteur effect you change the electron acceptor; you switch from oxygen to nitrogen, so that all the electrons which might go to oxygen go, in fact, to other metabolites. If you add glucose to aerobic cells, e.g. ascites cells, you switch from one type of endogenous substrate to another one, namely to glucose and its degradation products, by reason of the fact that you add glucose and, therefore, also glycolytic intermediates which had not been in the cells before (at least not in appreciable amounts). I don't think that we can expect the same kind of transition in metabolism in Pasteur's and our

conditions, despite the fact that some kind of steady state is finally reached, which might be equal or different for both conditions.

Lynen: I agree that if you study different conditions you will have different effects. I would emphasize that the control of fermentation by lack of ADP seems to me impossible if ATP is available at the same time. The phosphorylation of hexose by ATP would continuously regenerate ADP.

Hess: This is a question of the ratio between dephosphorylation and phosphorylation. In non-respiring ascites cells the ATP/ADP ratio is 1/1 and in respiring cells it is about 7/1 or even 10/1.

Lynen: I do not agree on this point. According to our analysis, and as far as I know, Prof. Holzer has had similar results, the ATP/ADP ratio does not change when you switch from nitrogen to oxygen. However, these determinations did not differentiate between cytoplasmic and mitochondrial ATP; the eventual transport of ATP from cytoplasm to mitochondria could not be detected by our method.



ENZYMIC REGULATION OF FERMENTATION IN YEAST CELLS

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THE analysis of the changes of metabolite concentrations which take place during the transition from one metabolic situation to another should give an insight into the co-operation of enzymes in living cells (Holzer, 1953, 1956). The following is a report on some results of the study of the co-operation of fermentative enzymes in living yeast cells by means of this method.

Regulation of fermentation rate by oxidation of reduced diphosphopyridine nucleotide

Only very low, barely demonstrable, concentrations of intermediates of carbohydrate metabolism can be found in yeast cells which have been starved by shaking with oxygen. If glucose is added to these starved yeast cells under aerobic or anaerobic conditions, then—almost without induction time—an intensive degradation of glucose commences, and readily measurable concentrations of carbohydrate metabolites are observed. We analysed such concentrations during the transition from the resting (starved) state to the stationary glucose-decomposing state (Holzer and Freytag-Hilf, 1959).

Fig. 1 shows the changes in fructose diphosphate concentration after the addition of glucose to starved yeast cells. Under aerobic and anaerobic conditions a characteristic "Einpendeln" of the fructose diphosphate concentration is observed (Holzer, 1956). Without doubt the occurrence of a maximum results from a change in the quotient:

$$Q = \frac{\text{production rate of the metabolite in question}}{\text{consumption rate of the metabolite in question}}$$

from a value greater than 1 to a value less than 1. With reversed signs, the same is valid for the occurrence of a minimum: compare for instance the ATP concentration in Fig. 2.

First, we have tried to find the causes for the occurrence of a maximum of fructose diphosphate concentration under anaerobic conditions. For this reason the determination of the concentration changes of fructose diphosphate, dihydroxyacetone phosphate, pyruvate, orthophosphate, adenosine

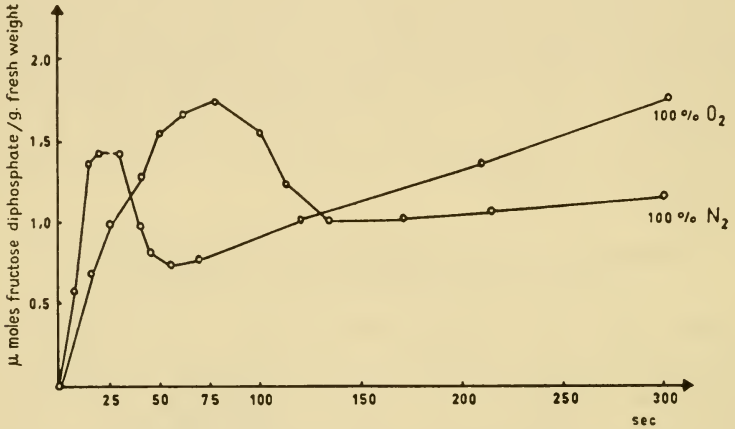

FIG. 1. Changes in fructose diphosphate after the addition of glucose to starved yeast cells (From Holzer and Freytag-Hilf, 1959. Reproduced by permission of the Editors, *Biochem. Z.*).

diphosphate (ADP), and adenosine triphosphate (ATP), as recorded in Fig. 2, has been carried out (Holzer and Freytag-Hilf, 1959; Holzer 1959). Fig. 2 shows that the concentration of dihydroxyacetone phosphate changes in perfect parallel with the concentration of fructose diphosphate. This proves that the enzyme aldolase occurs with surplus activity and does not play any rate-limiting rôle in the fermentation process. According to earlier observations, this is also true for the enzyme isomerase (Holzer and Holzer, 1953) since in glucose-metabolizing yeast cells the phosphoglyceraldehyde concentration, too, is always in equilibrium with the dihydroxyacetone

phosphate concentration. Hence, only an initial surplus production of fructose diphosphate due to the phosphorylation of fructose-6-phosphate or an initially too low dehydrogenation of phosphoglyceraldehyde phosphate by triose phosphate dehydrogenase comes into question for the occurrence of a maximum of the concentration of fructose diphosphate.

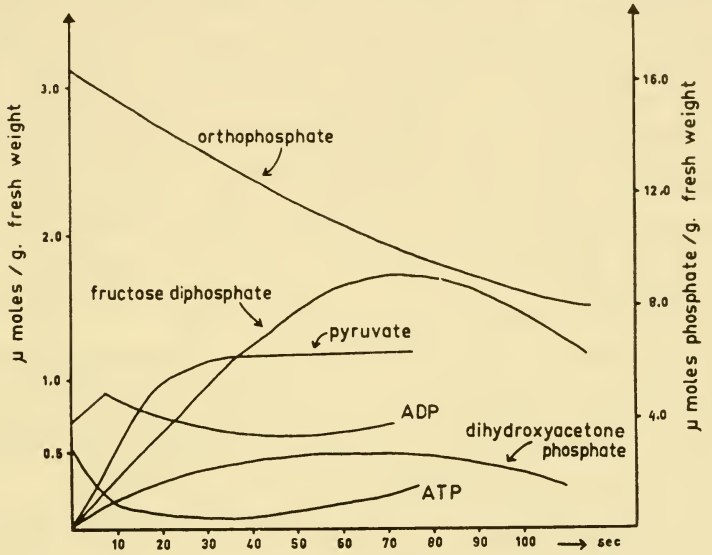

Fig. 2. Changes in metabolite concentrations after the addition of glucose to starved yeast cells under anaerobic conditions (From Holzer and Freytag-Hilf, 1959. Reproduced by permission of the Editors, *Biochem. Z.*).

These are the two enzymes, between the activities of which the substances fructose diphosphate, dihydroxyacetone phosphate and phosphoglyceraldehyde are found.

A reductive alteration of dihydroxyacetone phosphate to α-glycerophosphate need not be taken into consideration in yeast cells, since a specific enzyme for this reaction does not exist. This reaction is unspecifically catalysed by the enzyme alcohol dehydrogenase and takes place only if there exist unphysiologically high concentrations of reduced diphosphopyridine nucleotide (DPNH) and dihydroxyacetone phosphate (e.g. in the so-called sulphite fermentation).

For the explanation of the fructose diphosphate maximum a surplus phosphorylation of fructose-6-phosphate can be excluded because of the behaviour of the ATP concentration; the course of the ATP concentration is not at all correlated with that of the fructose diphosphate concentration, as is shown by Fig. 2.

Furthermore, the behaviour of the orthophosphate concentration shows that this substance, too, cannot have any share in the formation of a fructose diphosphate maximum by influencing the rate of triose phosphate dehydrogenation. In order to explain the fructose diphosphate curve, within the first 70 seconds a low and rate-limiting orthophosphate concentration, and after 70 seconds a high concentration of orthophosphate should be expected, thus promoting the degradation of fructose diphosphate via triose phosphate dehydrogenation. The observed behaviour of orthophosphate, however, is in complete contradiction to this.

A regulatory action of ADP upon triose phosphate dehydrogenation can be excluded for the reasons mentioned above with respect to ATP: the kinetics of the concentration changes of this substance are by no means correlated with the kinetics of the concentration changes of fructose diphosphate.

As a last possibility we have examined whether a lack of oxidized diphosphopyridine nucleotide (DPN), which is necessary for triose phosphate dehydrogenation, might be responsible for the accumulation of triose phosphate and fructose diphosphate. During the first minute after glucose addition to a yeast suspension, the concentration of acetaldehyde is so small that it cannot be determined with our usual methods (spectrophotometric test according to Warburg). This depends upon the fact that acetaldehyde is in equilibrium with alcohol, such that under anaerobic conditions only 1/200 of the alcohol concentration is present as acetaldehyde (Holzer, Holzer and Schultz, 1955). Moreover, the acetaldehyde formed within the cells diffuses rapidly into the medium, and for this reason cannot be concentrated in the cells in the same way as the phosphorylated metabolites (Holzer, Schultz and

Lynen, 1956). From this the assumption was obvious that a lack of acetaldehyde prevents a reoxidation of the DPNH formed by triose phosphate dehydrogenation, thus leading to the observed accumulation of fructose diphosphate. Fig. 3 shows that, in fact, this explanation is the right one. If glucose is added together with acetaldehyde to yeast cells

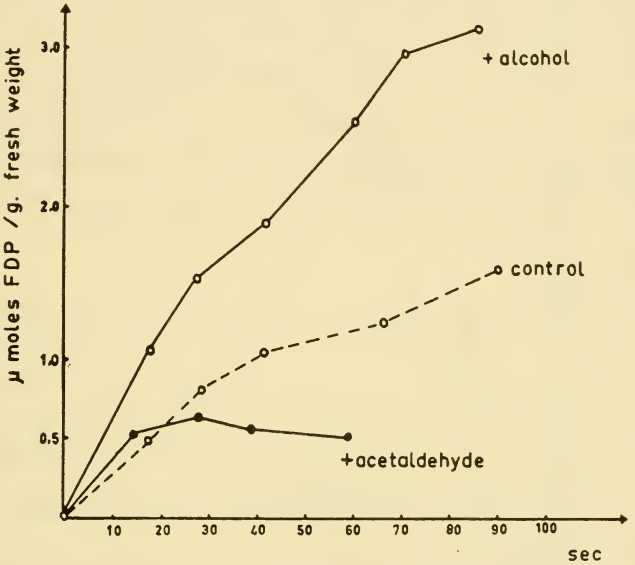

Fig. 3. Concentrations of fructose diphosphate (FDP) after the addition of glucose to starved yeast cells with and without alcohol and acetaldehyde (From Holzer and Freytag-Hilf, 1959: Reproduced by permission of the Editors, *Biochem. Z.*).

kept under anaerobic conditions, the characteristic accumulation of fructose diphosphate fails to occur. In other words, by the addition of an acceptor for the hydrogen of the reduced DPN the accumulation of fructose diphosphate can be prevented. If glucose is added together with alcohol, the reverse effect is found: the DPNH formed during triose phosphate dehydrogenation cannot transfer any hydrogen to acetaldehyde, because a strong "hydrogenating pressure" is exerted upon DPN due to alcohol dehydrogenase which is present

with high activity. This causes the fructose diphosphate concentration to increase still more than under normal conditions.

Finally, further evidence, showing that addition of acetaldehyde accelerates the triose phosphate dehydrogenation, is

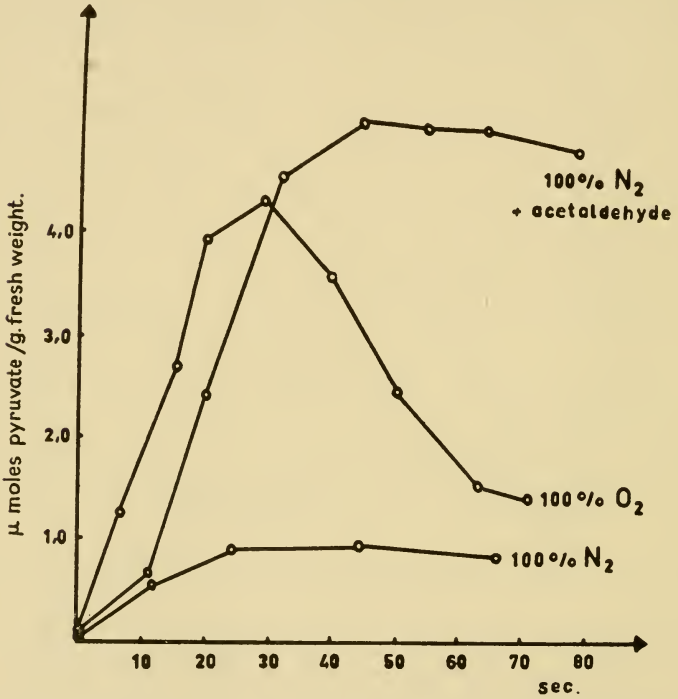

FIG. 4. Concentrations of pyruvate after the addition of glucose to starved yeast cells (From Holzer and Freytag-Hilf, 1959. Reproduced by permission of the Editors, *Biochem. Z.*).

obtained on examining the behaviour of the pyruvate concentration (Fig. 4). Without acetaldehyde, under anaerobic conditions a slow, constant rise of the pyruvate concentration takes place during the first 25 seconds, resulting in a value which, on the whole, remains constant. On the addition of acetaldehyde, however, a rapid increase of pyruvate is observed, undoubtedly depending on the fact that the decom-

position of glucose to yield pyruvate takes place so rapidly during the first seconds that the pyruvate decarboxylase cannot keep step with this supply. Moreover, the behaviour of the pyruvate concentration under aerobic conditions (Fig. 4) shows that under aerobic conditions, as well as on the addition of acetaldehyde under anaerobic conditions, pyruvate accumulates rapidly. It seems that in the presence of oxygen there is no inhibition of triose phosphate dehydrogenation due to a lack of oxidized DPN, since DPNH now can transfer its hydrogen to oxygen.

In summary, it may be said that all measurements shown in Figs. 1–4 agree with the assumption that a lack of oxidized DPN limits the rate of fermentation during the first seconds after the addition of glucose to starved yeast cells.

Increase of aerobic fermentation by addition of NH_4^+ ions to glucose-oxidizing yeast cells

If NH_4^+ ions are added to glucose-oxidizing yeast cells, soon a strong increase of aerobic fermentation, and after some time (in the presence of the necessary growth factors) the occurrence of growth and cell multiplication, are observed. In connexion with studies on carbohydrate metabolism in growing cells and tissues we have been interested in the mechanics of how the rise in aerobic fermentation following the addition of NH_4^+ ions comes about. Fig. 5 shows the changes in the concentration of orthophosphate, pyruvate, acetaldehyde and α-ketoglutarate after the addition of NH_4^+ ions to glucose-oxidizing yeast cells (Holzer, 1959, 1958; Holzer and Witt, 1958). From these measurements we conclude that the following sequence of events occurs: at first α-ketoglutarate is aminated reductively to glutamate by means of DPNH and reduced triphosphopyridine nucleotide (TPNH), respectively —the two glutamate dehydrogenases can be concentrated and separated from each other (Holzer and Schneider, 1957); the lack of α-ketoglutarate causes an inhibition of respiration as well as oxidative phosphorylation, by which the observed

rapid rise of the orthophosphate concentration is brought about. The increase in the orthophosphate concentration gives rise to an accelerated triose phosphate dehydrogenation followed by an increase in the pyruvate and acetaldehyde

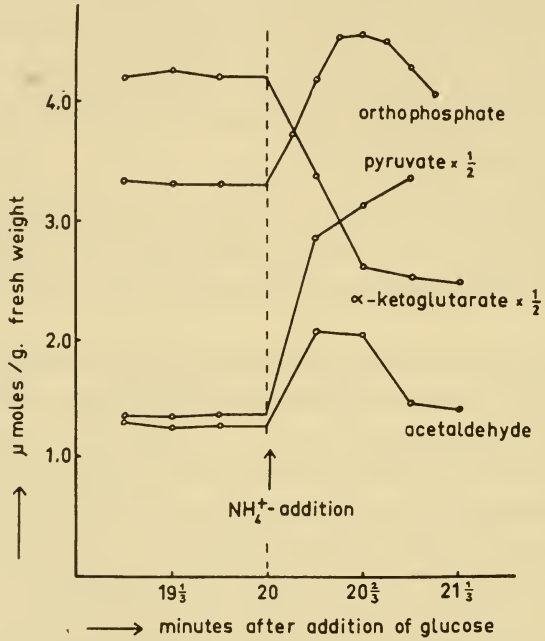

FIG. 5. Changes in concentrations of carbohydrate metabolites after the addition of NH_4^+ ions to glucose-oxidizing yeast cells. (From Holzer and Witt, 1958*a*. Reproduced by permission of the Editors, *Biochem. Z.*)

concentration (Fig. 5). The accelerated pyruvate decarboxylation is revealed manometrically as an increase in aerobic fermentation.

After the rapid rise of orthophosphate concentration there is a rapid drop to small concentrations. During this latter period the orthophosphate concentration cannot be responsible for the increased aerobic fermentation which lasts for a long time after the addition of NH_4^+ ions. At present we are attempting to solve the problem of whether this may be

attributed to a change in the distribution of the available phosphorus between orthophosphate and bound phosphates.

Reoxidation of reduced triphosphopyridine nucleotide, a bottleneck in the oxidative pentose phosphate cycle (Holzer and Witt, 1958*b*)

The analysis shown in Fig. 6 reveals the presence of another regulative mechanism which starts after NH_4^+ ions have been added to glucose-oxidizing yeast. Besides the changes

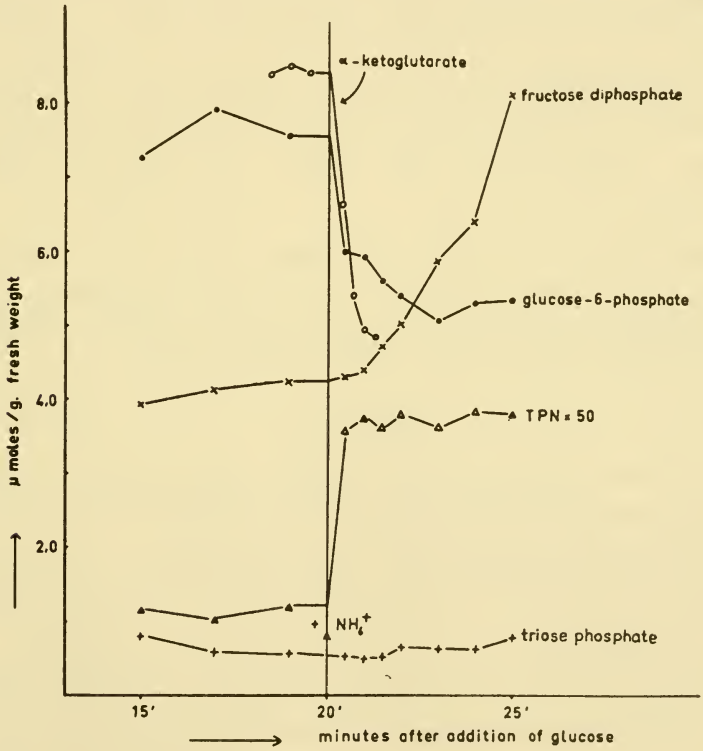

FIG. 6. Changes in concentrations of TPN, glucose-6-phosphate, and other metabolites after the addition of NH_4^+ ions to glucose-oxidizing yeast cells (From Holzer and Witt, 1958*b*. Reproduced by permission of the Editors, *Angew. Chem.*).

already shown in Fig. 5, a rapid decrease of the glucose-6-phosphate concentration and a rapid increase of the concentration of oxidized TPN have been observed. We ascribe this to the fact that in yeast cells as well as in animal tissues the dehydrogenation of glucose-6-phosphate caused by the "Zwischenferment" is limited by a lack of oxidized TPN, as most of the TPN is present in its reduced form. On the addition of NH_4^+ ions, TPNH is used up for the reductive amination of α-ketoglutarate, thus yielding oxidized TPN, which now makes possible the dehydrogenation of glucose-6-phosphate. This starts the oxidative pentose phosphate cycle. Since the dehydrogenation of glucose-6-phosphate leads to an accumulation of pentoses, here is a reasonable mechanism by means of which the NH_4^+ ions, which are causing growth, promote the formation of pentoses and therefore of ribonucleic acids, the latter being necessary for growth. In experiments with extracts from yeast and liver we could demonstrate that an activation of the pentose phosphate cycle by NH_4^+ ions as mentioned above actually takes place, leading to an accumulation of pentoses (Witt and Busch, 1958, unpublished). At present we are investigating the *in vivo* operation of this mechanism, with the help of ^{14}C-labelled glucose.

Summary

(1) Some problems of the enzymic regulation of carbohydrate metabolism in yeast cells have been investigated by measuring changes in metabolite concentrations.

(2) During the first minute after the addition of glucose to starved yeast cells the degradation of glucose is limited by lack of oxidized DPN, i.e. by a too slow reoxidation of DPNH.

(3) The origin of the aerobic fermentation, appearing after the addition of NH_4^+ ions to glucose-oxidizing yeast cells, may be explained by a regulation via the adenylic acid system.

(4) It has been shown that in glucose-oxidizing yeast cells the direct oxidation via the pentose phosphate cycle is limited by a lack of oxidized TPN. Addition of NH_4^+ ions

causes TPNH oxidation due to the amination of α-ketoglutarate, thus supplying TPN and activating the pentose phosphate cycle.

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DISCUSSION

Coxon: Foulkes (1953, *Biochem. J.*, **54**, 323) described a factor which he called the citrate oxidation factor. This was a dialysable material that he got from yeast, having the property that when added to yeast which was oxidizing pyruvate it minimized the quantity of citrate which accumulated. A good deal of analytical work was subsequently done to characterize this factor, and it was found (Whitehouse, M. W., Kent, P. W., Peters, R. A., and Foulkes, E. C. (1954). *Biochem. J.*, **58**, 437) that most of the activity could be explained by ammonium ions in the extract—which fits rather well with Prof. Holzer's work.

Racker: Prof. Holzer, in your Fig. 2 the curves for fructose diphosphate and triose phosphate were parallel, though of course the triose phosphate values were much lower. In Fig. 6 the fructose diphosphate values went up while triose phosphates did not. I am sure there is a good reason for this but I cannot think of one.

Holzer: Since in the experiment shown in Fig. 6 the concentrations of fructose diphosphate and triose phosphate are remarkably higher than in the experiment shown in Fig. 2, and since the equilibrium constant of zymohexase depends upon the concentration, it is understandable that triose phosphate—with respect to fructose diphosphate—changes less in Fig. 6 than in Fig. 2.

Hess: The fructose diphosphate surplus which Prof. Holzer has observed in yeast as a consequence of a lack of DPN can also be demonstrated in ascites tumour cells after addition of glucose, especially if

iodoacetate is added in order to simulate the lack of DPN. This condition is illustrated in Fig. 1, which shows the kinetics for fructose

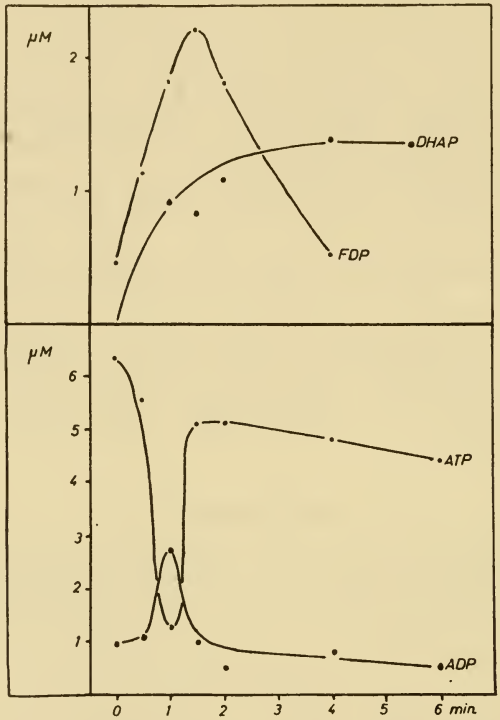

FIG. 1 (Hess). Steady state change of concentration during transition from endogenous to glucose metabolism in the presence of 1·7 mM iodoactate. Concentrations are given in μM/g. wet weight. Glucose, 0·75 mM.

diphosphate, dihydroxyacetone phosphate, adenosine diphosphate and triphosphate.

Chance: Since the lack of DPN has been postulated to be the cause of this metabolic control and since we have for some time been studying the pyridine nucleotide kinetics in yeast cells following the addition of glucose to the starved cells, it is possible that our data may shed some light on the question raised by Prof. Holzer's results and by Dr. Racker. The typical response of reduced pyridine nucleotide to glucose addition under aerobic and anaerobic conditions is shown in Fig. 1A and B.

Under our experimental conditions, DPN reduction starts within a few seconds after addition of glucose and reaches a maximum at 18 seconds. Thereafter there is an oxidation of pyridine nucleotide resulting in a steady state concentration of DPN very nearly as high as it was prior to the addition of glucose. We can state, therefore, that there is no

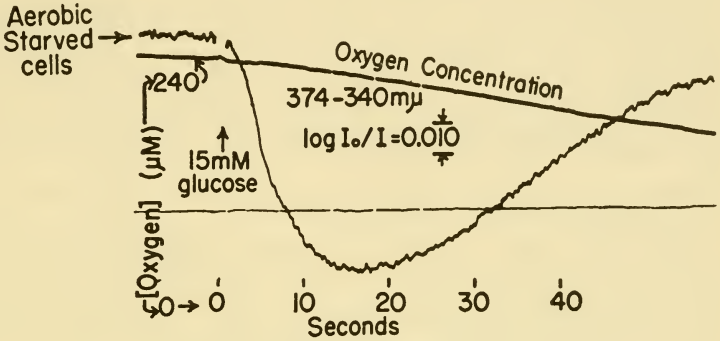

FIG. 1A (Chance). (Expt. no. 177)

lack of oxidized DPN in the cell at this time which would limit fermentation in the way illustrated by Prof. Holzer's Figs. 1 or 2. A similar conclusion can be drawn with regard to the addition of glucose under anaerobic conditions (Fig. 1B) where even in the presence of 20 mM

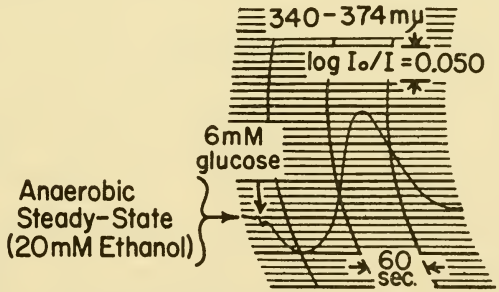

FIG. 1B (Chance). (Expt. no. 321c)

ethanol the characteristic fluctuations of the steady state of reduced pyridine nucleotide result in considerably more DPN in the cell than was present prior to the addition of glucose. It seems, therefore, that the theory of a DPN limitation of fermentation one or two minutes after the addition of glucose is without direct experimental support on the basis of the assay of the total DPN within the cell; if, indeed, DPN

is postulated to be rate-limiting in this process and one must invoke a hypothesis of localization of DPN as well as that already presented earlier on ATP localization (Lynen, F., and Koenigsberger, R. (1951). *Justus Liebigs Ann. Chem.*, **573**, 60). (See also p. 256).

Racker: Was the DPN measured by the absorption given by the triose phosphate dehydrogenase-DPN compound?

Chance: The DPN assay of Fig. 1A was based on the assumption that the starved cell contained nearly all its pyridine nucleotide in the oxidized form. In other experiments the DPN is measured by the amount of complex (Racker, E., and Krimsky, I (1952). *J. biol. Chem.*, **198**, 731; Chance, B. (1954). *In* The Mechanism of Enzyme Action, p. 444, Ed. McElroy, W. D., and Glass, B. Baltimore: Johns Hopkins Press) that disappears upon the addition of iodoacetate to the starved yeast cell.

Racker: What happens when DPNH disappears? Does acetaldehyde act as acceptor?

Chance: The rapid oxidation of pyridine nucleotide following its initial reduction upon glucose addition is probably due to α-glycerolphosphate dehydrogenase activity; addition of glucose to cells pretreated with iodoacetate and ethanol causes a rapid oxidation of reduced pyridine nucleotide. While it is possible that a system other than α-glycerolphosphate dehydrogenase could be responsible, none is known that could act under these conditions. In the absence of iodoacetate, acetaldehyde accumulation could also contribute to this oxidation reaction and Prof. Holzer's assays for acetaldehyde accumulation in the first 30 seconds after glucose addition also favour this view.

Now returning to Dr. Racker's question on ammonia and to Prof. Holzer's proposed explanation for the increase of aerobic fermentation caused by the addition of NH_4^+, it is worth while to consider not only the effect of NH_4^+ on the α-ketoglutarate equilibrium but also the steady state concentration of pyridine nucleotide. It is known that ammonia and amines penetrate the yeast cell and cause changes of intracellular pH which can be studied with suitable indicators (Brand, K. M. (1945). *Acta physiol. scand.*, **10**, suppl. 30). In fact, we have attempted to compute the equilibrium constant for the following chemical reaction on the basis of titrations of intracellular pyridine nucleotide with acetaldehyde and alcohol.

$$H^+ + \text{DPNH} + \text{acetaldehyde} = \text{DPN}^+ + \text{ethanol}$$

Fig. 2A provides a verification of the shift of the steady state of reduced pyridine nucleotide towards reduction by increasing concentrations of ammonia. In this particular experiment the yeast cells were suspended in an alkaline phosphate medium of pH 9·3 in order to produce an optimal effect of the ammonia. In addition, the oxidation-reduction state of the pyridine nucleotide was set at a relatively oxidized level by an alcohol-aldehyde "redox buffer" (23 mM and 1·5 mM, respectively). Aliquots of a solution of 1·5 M ammonia were added to the anaerobic cells. The effectiveness of ammonia in producing increased reduction of intracellular pyridine nucleotide is clearly demonstrated.

Not only does the addition of ammonia shift the steady state of reduced pyridine nucleotide but it also has a dramatic effect upon the oxidation-reduction levels of the cytochromes and upon the respiratory

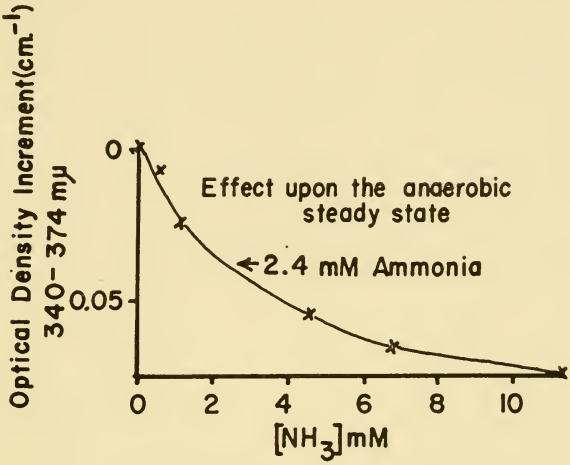

FIG. 2A (Chance). (Expt. no. 175)

rate. This is illustrated in Fig. 2B which represents conditions similar to those of Fig. 2A except that the cells are aerobic and respiration was

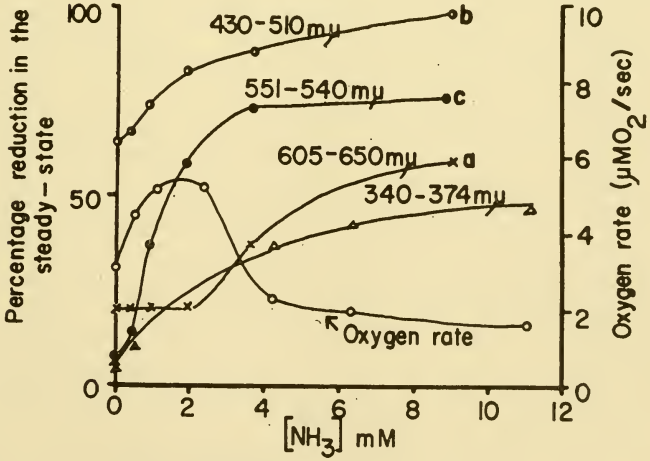

FIG. 2B (Chance). (Expt. no. 175b, c)

measured. We observe that low ammonia concentrations stimulate respiration but higher concentrations inhibit. The effect upon the cytochromes is a striking one. Whereas in the absence of ammonia, cytochrome *c* (551–540 mμ) is very little reduced in the steady state, low concentrations of ammonia cause reduction to the point where cytochrome *a* (605–650 mμ) is also affected, with the consequent reduction of respiratory rate. It is our conclusion that ammonia causes an unknown type of inhibition of electron transfer *in* the respiratory chain. Furthermore, the increased reduction of pyridine nucleotide (340–374 mμ) shows clearly that it is not lack of substrate that causes the respiratory inhibition; more DPNH is present in the presence of ammonia than in the absence of it. In view of these results, it seems scarcely reasonable that the effect of NH_4^+ upon the α-ketoglutarate equilibrium is the sole cause of respiratory inhibition.

On the other hand, the effects of ammonium chloride upon the steady state level of pyridine nucleotide in mitochondria which are freely permeable to this ion, have been studied (Chance, B., and Williams, G. R. (1956). *Advanc. Enzymol*, **17**, 65) and it is found that a system buffered with α-ketoglutarate-glutamate "redox buffer" will respond to m-molar concentrations of ammonium chloride with an oxidation of reduced pyridine nucleotide and an inhibition of respiration. It is apparent, however, from the data presented in Fig. 2 that the metabolic regulation in the intact yeast cell is a more complex one and is probably inadequately explained by the hypothesis presented by Prof. Holzer.

Holzer: The changes in the rate of oxygen uptake, as measured after the addition of ammonium ions by Prof. Chance, are in accordance with our experiments: we worked with approximately 80 m-moles NH_4^+ per litre, i.e. in a concentration range in which Prof. Chance also observed an inhibition of oxygen uptake. The results concerning the state of reduction of DPN are in contrast to our findings: from analyses of the concentrations of alcohol and acetaldehyde we have calculated a diminution of the ratio DPNH/DPN after the addition of NH_4^+ ions (Holzer, Holzer and Schultz, 1955, *loc. cit.*; Holzer, Schultz, and Lynen 1956, *loc. cit.*), whereas Prof. Chance observes an increase in the ratio DPNH/DPN. This discrepancy might be due to the following: (1) our experiments were carried out at pH 6, whereas Prof. Chance works under alkaline conditions; (2) we measured—via the determination of alcohol and acetaldehyde by means of the alcohol dehydrogenase equilibrium—the ratio of the concentrations of the free dissociating pyridine nucleotides, whereas, Prof. Chance—using the spectroscopic method—determines the sum of both free and bound pyridine nucleotide.

When DPNH is assayed with a method comprehending bound as well as free DPNH (by killing the cells with hot NaOH and subsequent determination of DPNH by means of alcohol dehydrogenase and acetaldehyde), then we, too, find an increase in DPNH immediately after the addition of NH_4^+ to glucose-oxidizing yeast cells (Holzer, H., and Witt, I., unpublished experiments).

Chance: You did not monitor your intracellular pH? It is possible that the effect could be reversed by the addition of acid.

King: In the system with ammonium salt and glucose, have you observed any net growth of yeast?

Holzer: Not in our experiments, because we only looked at what happened after a few minutes; but after 30 to 60 minutes one can observe growth.

Chance: We never tried to observe growth.

King: Then I do not understand how you define the steady state there.

Racker: Perhaps you could tell us how you define growth?

King: Growth is the net increase in cell mass.

Holzer: If you wait for 30 minutes, you may get an increase of 3 to 5 per cent, and if you wait longer you may get the well known exponential growth. What we have studied is what happens in the first few seconds and minutes, and from this we hope to deduce how growth is initiated by metabolic alterations. Therefore, our interest is focused on what happens *before* you see an increase in cell mass.

King: If we use a bacteriological term, then your study is still at the lag phase?

Holzer: Yes, that is right.

Hinshelwood: When you add the ammonium salt to a yeast preparation, one of the most important points biologically is that the system is preparing for synthesis, growth and division. After some finite delay, mass will increase, and division will set in. The two will not be phased exactly together. It would be extremely interesting if these experiments were carried to the stage where the first mass increase is observed so that one could see which of the processes reaches a critical level and triggers off something else. Presumably all the processes have been evolved so as to fit cells optimally to grow and form new material. It is interesting to note that a bacterial suspension which is reducing nitrate vigorously, is completely inhibited for a long period if ammonium salts are added. The explanation is fairly simple, and is an interesting example of regulation: in the presence of ammonia, a much more effective process goes on, which consumes the carriers much more rapidly and reduces their concentrations to a level where they cannot reduce nitrate.

Magasanik: To introduce a note of caution, as far as the experiments with ammonium salts are concerned: we have just by chance come across two DPN-linked dehydrogenases, which require ammonium ion for activity. One is inosinic acid dehydrogenase, where the ammonium ion can be replaced by potassium. The other one is a glycerol dehydrogenase in *Aerobacter aerogenes*, where ammonium ion is much more effective than potassium. The affinity is low; in other words, ammonium salt has to be added to a high level. The ammonium ion may thus act as an activator for dehydrogenase which in turn may bring about changes in DPN levels.

Lynen: To go back to the pH effect, Prof. Chance, did you add free ammonia or ammonium chloride?

Chance: Ammonium chloride. Dr. Magasanik, is the effect which you describe reversed by hydrogen ions?

Magasanik: No.

Slater: Prof. Chance, what do you mean by reversed effect?

Chance: We can titrate the steady state of pyridine nucleotide back and forth between reduced and oxidized, by acid and base. It is well known that ammonia and amines penetrate the yeast cell and are only slowly pumped out.

Slater: Then it should be possible to study this effect of ammonia at constant pH.

Chance: We did study this at constant pH, but you can get rid of it by lowering the pH.

Krebs: Dr. Magasanik, were the ammonia concentrations, to which you referred, really comparable in level to those which one might expect in the cell?

Magasanik: I think so. Dr. Lin worked on this as far as the glycerol dehydrogenase is concerned. We have published the figures on inosinic acid dehydrogenase (Magasanik, B., Moyed, S. H., and Gehring, L. B. (1957). *J. biol. Chem.*, **226**, 339).

Lynen: Dr. Holzer, did you measure the time-course of the disappearance of ammonia?

Holzer: It is a straight line for a long time. The disappearance of α-ketoglutarate explains about 60 to 90 per cent of the disappearance of ammonium ions in the first seconds. The remaining 10 to 40 per cent of the ammonium ions are converted perhaps to carbamyl phosphate, or participate in other ammonium-fixing reactions.

Krebs: These are likely to be other ammonium-binding reactions, but I take it that you consider this to be the most important one, and that the other amino acids are all formed by transamination.

MECHANISMS FOR CONTROL OF ENZYME SYNTHESIS AND ENZYME ACTIVITY IN BACTERIA

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Regulation of cell metabolism will be considered here with respect to a set of reactions which differ from those dealt with in the preceding communications. The difference in outlook stems from the use of bacteria, which can double their mass in as little as eighteen minutes. This rapid growth draws attention to problems of regulation of the numerous reactions of biosynthesis, in contrast to problems of energy supply, which are more obvious in more slowly growing cells.

Synthetic reactions take place in a highly organized way. The organization in bacteria functions as if the available nutrients were utilized so as to permit as rapid a growth as possible under given conditions. Bacteria grow more rapidly as the medium in which they are suspended is made progressively richer in amino acids, purines, pyrimidines, etc.; therefore, those nutrients not supplied in the medium must somehow be made at different rates, sufficiently rapid in each medium to keep up with the increased demands of growth. Conversely, when the medium is made less complete, the rate of bacterial growth is decreased, and the reactions now are adjusted to the new requirements (Novick and Szilard, 1954). Results such as these show that control mechanisms exist for organization of metabolism.

Control as considered here is illustrated by the following quantitative example. *Escherichia coli* can use only glucose plus a few salts, in aerobic growth, to make a multitude of products. The utilization of carbon goes on in a very efficient way: only small quantities of the metabolic intermediates (amino acids, nucleotides, etc.) spill out of the cells or are

found inside them, perhaps 10 per cent of the amount incorporated (Roberts *et al.*, 1955). Therefore, on the average the reactions are controlled to run within about 10 per cent of the minimum necessary rate. One naturally asks how such a controlled system operates.

The aim of the present paper is to discuss the extent to which recent discoveries permit a description of the integration of metabolic processes. Bacterial metabolism is not rigidly fixed but is made up in a very flexible way, so that as conditions are changed the reactions adjust and attain a new balance with their surroundings in order to permit growth. Therefore, one must seek mechanisms which accelerate the rates of individual reactions when their reaction products are not made as rapidly as other metabolites, and other (or similar) mechanisms which lower the supply of these products when they are in excess over the demands of overall growth. For the greatest possible economy of the cell it would be desirable to prevent the formation of surpluses of two sorts of products, (i) metabolic intermediates and (ii) large molecules, such as enzymes. One might hope to find mechanisms which prevent formation of surpluses of each of these kinds of products of cell metabolism. Several such mechanisms have indeed been described recently, and some examples will be noted below. In conclusion, some comments will be made regarding the manner in which these mechanisms may function. The subject has recently been discussed extensively (Lwoff, 1957; Magasanik, 1957; Pardee, 1958).

Types of regulation

Metabolic requirements can be expressed in terms of changes in the concentrations of certain small molecules inside the cells; these, in turn, might regulate the rates of key reactions in the metabolic pathways. Small molecules may have two general kinds of effect on enzymes: they can alter the rate of synthesis of an enzyme, or they can modify the rate at which an enzyme molecule functions. In both cases the effect of the small molecule can either be positive or negative. One of

these four effects is well understood in principle: the increase in rate of enzyme activity as substrate concentration is increased. Implications for metabolism have been discussed at length (Dixon, 1949) and will not be commented upon further here. The next sections will deal with experimental examples of the other three cases: inhibition of enzyme activity, induction and repression of enzyme synthesis.

Enzyme inhibition

Inhibition of a reaction by one of its products would be most advantageous for preventing surplus production of

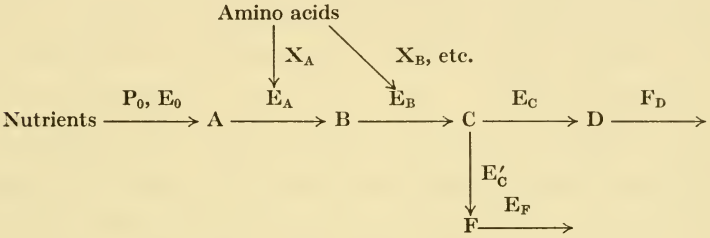

FIG. 1. Schematic representation of a metabolic pathway. P_0 is a permease; A,. . . . F are metabolites; E_0, E_A,. . . . E_F are enzymes; and X_A, X_B are the "systems" for synthesis of E_A, E_B, etc.

metabolites. Especially useful would be an inhibition of an early reaction of a metabolic sequence by a product near the end of the sequence: such a "feedback inhibition" would serve to prevent production of any intermediate in the entire sequence when excess product is present. In Fig. 1, the inhibition of the conversion of A to B by D would represent such a case.

Several examples of feedback inhibition have been found in bacteria. A case which has been extensively studied is that of the inhibition of the pathway of pyrimidine synthesis (Yates and Pardee, 1956). The existence of this feedback control, active during metabolism, was discovered in the course of studies on a mutant of *Esch. coli* that required a pyrimidine such as uracil for growth. This mutant possessed the enzyme

necessary for synthesis of orotic acid (an intermediate of pyrimidine synthesis), but could not convert orotic acid to pyrimidines. It was observed that orotic acid was not produced by the bacteria while they were growing in the presence of uracil. But as soon as the uracil was used up, orotic acid and the two intermediates of orotic acid synthesis were found in the medium. Clearly, some condition in the growing bacteria blocked the first specific step of the metabolic pathway. Further experiments with whole cells showed that it was the presence of a pyrimidine that inhibited the appearance of these compounds. Finally, it was shown with cell-free preparations of the organism that a product at the end of the sequence (cytidylic acid) was quite a good inhibitor of the first specific step, i.e. the condensation of aspartate and carbamyl phosphate. The inhibition is competitive; and therefore the activity of the enzyme *in vivo* must depend on a balance between substrates and inhibitor. This inhibition is direct, and not an equilibrium situation in which the reaction is inhibited by a surplus of products acting through all of the intermediate steps, as shown by the *in vitro* result and also by the inhibition *in vivo* in mutants which lack intermediate enzymes.

Feedback inhibitions have been discovered for a number of other pathways including those for isoleucine (Umbarger, 1956), purines (Gots, 1957) and threonine (Wormser and Pardee, 1958). Also, numerous observations regarding limited production of metabolic intermediates by mutant bacteria (see Novick and Szilard, 1954), and utilization of nutrients supplied in the medium preferentially to endogenous products (Roberts *et al.*, 1955) suggest feedback controls. In general, these data do not permit one to distinguish between inhibition of enzyme activity and inhibition of enzyme formation (repression), to be described below.

Enzyme induction

It is well known that the concentrations of some enzymes in bacteria are not constant but depend very strikingly upon

conditions of growth. The rate of synthesis of an inducible enzyme can be stimulated as much as several thousand fold upon the addition of its substrate or a related compound to the medium (Cohn and Monod, 1953). The synthesis of other enzymes can be influenced less directly, by a process called sequential induction (Stanier, 1950). In sequential induction the added compound (A, Fig. 1) induces an enzyme (E_A) which converts it to a second compound (B); the second compound induces a second enzyme (E_B) which converts it to a third compound (C); (C) induces a third enzyme (E_C), etc. Induction thus influences the concentrations of enzymes that are not directly involved with materials in the medium. By this means, enzymes in metabolic pathways could be maintained in sufficient excess to keep the concentrations of their substrates at low levels.

Another set of activities in bacteria are inducible; these are mechanisms ("permeases") for the transport of sugars, amino acids and other metabolites into the bacteria (Cohen and Monod, 1957). Permeases are capable of specifically building up very high concentrations (up to several per cent of the dry weight of the cell) of their substrates inside the bacteria. The function of inducible permeases and inducible enzymes in regulation of metabolism will be considered below.

Enzyme repression

Repression, a mechanism just the opposite of induction, controls the concentrations of some bacterial enzymes. In repression, some small molecule inhibits the formation of an enzyme; and often the latter is an enzyme that acts at an early stage in the metabolic sequence of which the repressor is a product (e.g. in Fig. 1, D might inhibit the process catalysed by X_A). Some early examples of repression, which were not investigated extensively, were the repression of "methionine synthase" by methionine (Wijesundera and Woods, 1953; Cohn and Monod, 1953) of tryptophan desmase by tryptophan (Cohn and Monod, 1953), of β-galactosidase by galactose (Cohn and Monod, 1953), and of the terminal transaminase in

the valine pathway by valine (Adelberg and Umbarger, 1953). More recently, arginine has been found to repress formation of two enzymes in its pathway of synthesis: acetyl ornithinase (Vogel, 1957) and ornithine transcarbamylase (Gorini and Maas, 1957). In the latter work, use of the chemostat for continuous culture at low repressor concentration points the way to a most useful technique for the study of repression. An enzyme of purine biosynthesis is repressible (Magasanik, 1957). Also, uracil was found to inhibit the formation of the first three enzymes of the pathway of pyrimidine synthesis; activity of the first enzyme, aspartate carbamyl transferase, was increased 500 times by release of the repression (Yates and Pardee, 1957). The action was shown to be a repression rather than an inhibition by a feedback mechanism of formation of inducers (which would have the same final effect). Also it was shown that repression controlled the synthesis of the enzyme from amino acids, rather than that it took part in some sort of activation phenomenon. This repression of the first specific enzyme of the pathway by an end product six or more steps removed would appear particularly suited for metabolic control.

It now appears that enzyme induction and enzyme repression are expressions of the same phenomenon. The genetically alternative situation is constitutivity, i.e. the production of an enzyme at a rate independent of the presence of small molecules acting as inducers or repressors. Recent experiments indicate that genetically constitutive bacteria lack a repressor for β-galactosidase synthesis. This repressor is synthesized in inducible cells (Pardee, Jacob and Monod, 1958); and added inducer serves to overcome the repression. Thus, it would appear that inducible (and repressible) enzymes might be formed in amounts which depend on a balance between inducers and repressors.

Metabolic regulation

The experimental observations described above will now be used to suggest a mechanistic scheme for control of bacterial

metabolism. The description will be arbitrarily divided into three parts: (*a*) uptake of nutrients, (*b*) intermediate steps of metabolism and (*c*) formation of end products (large molecules).

(*a*) *Uptake of nutrients.* The initial steps of metabolism are affected by processes which act so as to provide a plentiful supply of nutrients. Permeases furnish a high intracellular concentration of substrates, thus permitting subsequent enzymes to function at more rapid rates than if they had available only the lower concentrations of substrates in the medium. Induction mechanisms tend to form large quantities of permeases and of enzymes, and consequently provide for high rates of metabolism. For instance, conditions have been found which either do not permit formation of β-galactosidase or else permit it to be formed only at the maximal rate in an individual bacterium (Novick and Weiner, 1957).

Mechanisms which limit the rate of utilization of nutrients are not so well known, but there are good indications of their existence. Diauxie provides an example: if the bacteria are provided with two energy sources, one may be utilized in preference to the other (a situation somewhat analogous to the Pasteur effect). One imposes a limitation on the other. A second case is observed when bacteria are transferred to a new medium containing lactose as the sole carbon source; they synthesize the enzyme for lactose utilization in preference to the other proteins of the cell (Pardee, 1955; Rickenberg and Lester, 1955; Mandelstam, 1957). This result could imply that the capacity for synthesis of this enzyme was not fully utilized under more favourable growth conditions. Mechanisms whereby nutrient limitations can be achieved are discussed later in this symposium (Magasanik, p. 334).

(*b*) *Intermediate steps of metabolism.* Reactions of intermediary metabolism can be divided into two classes in regard to regulation. The fact that formation of metabolic intermediates takes place in a series of steps means that each reaction is dependent on the preceding ones for its supply of substrates. Therefore, mechanisms for restricting the rates of

reactions are not necessary if the rates must be equal to the rates of the preceding reactions (i.e. wherever their enzymes are present in adequate amounts, perhaps provided through induction mechanisms). Certain steps of the pathway ("pacemaker reactions") must somehow be controlled. First, the end product of a series of reactions must somehow be limited so as to co-ordinate with products of other pathways. Second, if two pathways diverge from some compound (as from C in Fig. 1), some regulation of the partition of the compound between these sequences is required [alternative metabolic pathways (Potter and Heidelberger, 1950)]. Feedback inhibition at the first specific step of a sequence could provide an adequate means of control, since the rate of the entire pathway would depend on the accumulated end product. Examples of the validity of this idea are found in the observation that an amino acid provided in the medium usually prevents its own synthesis by *Esch. coli:* the amino acids formed *de novo* (from a radioactive carbon source) are found neither in the bacterial proteins nor in the medium nor inside the cells under these conditions (Roberts *et al.*, 1955).

Enzyme repression could also function to limit pacemaker reactions. In one case repression might act, and in another feedback inhibition could serve. The time required for these actions to be effective must be quite different, however. Whereas inhibition should act instantaneously, repression must have a more gradual action. Although the latter commences immediately, some time would be required to increase the enzyme concentration and especially to decrease an existing concentration of enzyme (by growth without enzyme synthesis).

(*c*) *Formation of end products.* Large molecules (such as enzymes) should not be synthesized in excess of the quantities that are required for growth, for efficient functioning of bacteria (since the formation of a large molecule must require energy). Thus, for example, a bacterium could have a selective advantage if it did not have to make β-galactosidase when no galactosides were available. Repression and induction thus

play a dual rôle in control of metabolism: they regulate the formation of small molecules, and also provide for an economical formation of certain large molecules (which happen to be enzymes).

The syntheses of proteins and nucleic acids, processes in which many metabolic pathways converge, provide outstanding possibilities for metabolic control. Furthermore, since these materials make up a large part of the organic material of bacteria (perhaps 80 per cent), a means might be available for the co-ordination of the bulk of the metabolism of the bacteria. First, it must be remembered that proteins (or even polypeptides) are synthesized only when all of their amino acids are present (Spiegelman, Halverson and Ben-Ishai, 1955). All amino acids whose pathways are controlled by feedback or repression mechanisms would be formed at rates which equal their rates of removal into proteins; this, in turn, would depend on the rate of supply of the least rapidly available amino acid.

Finally, it may be noted that the syntheses of proteins and nucleic acids are closely related in bacteria: one does not commonly find the synthesis of one of these macromolecules continuing when a component of the other is withheld (Pardee, 1954; Spiegelman, Halverson and Ben-Ishai, 1955; Pardee and Prestidge, 1956; Gros and Gros, 1958). Therefore, if one component, say a nucleic acid precursor, were formed more slowly than any amino acid, it would limit the synthesis of most large molecules, and consequently of many of the small molecules.

Summary

The small molecules in bacteria can regulate enzymes in four ways: by stimulation of activity, inhibition of activity, stimulation of formation (induction) and inhibition of formation (repression). Products of metabolic sequences can act on early reactions or enzyme syntheses (feedback), so that supply is adjusted to demand for incorporation into large molecules. The rates of many synthetic pathways could be co-ordinated

through inter-related syntheses into large molecules; and the rates of syntheses of the latter might be determined by the supply of least readily available building blocks.

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DISCUSSION

Racker: Dr. Pardee, would you say that in the wild-type organism there is anywhere close to 5×10^{-3} M cytidylic acid accumulation? Does the intracellular concentration ever exceed the K_i value?

Pardee: If synthesis were not shut off, cytidylic acid would accumulate

very rapidly. I do not have the data with me, but there is a measurable concentration of cytidylic acid, a measurable pool, within the cell.

Racker: For a complete inhibition of the enzyme it would be necessary to reach values at least 5 times that of the K_i value. This would require a concentration of over 20 μmoles per ml. Do you think that so much accumulates?

Magasanik: We have measured the levels of the intracellular nucleotides in bacteria. Prof. Potter, you could give the answer, because our results were very close to those which you obtained in animal cells.

Potter: The cytidylic content is very small, much smaller than the adenylic and the uridylic levels. But one cannot even begin to disqualify the validity of the general line of argument in that way.

Racker: I agree with you. We have been postulating that compartmentation is contributing to the Pasteur phenomenon in ascites tumour, and we could invoke compartmentation here, too. But since this is a difficult thing to prove, we are better off if we eliminate other possibilities first.

Magasanik: Cytidylic acid is probably not only a nucleic acid precursor but also a coenzyme; therefore, it should accumulate to some extent.

Pardee: We worried about this at the time we did the work and we came to the conclusion that it was not very important. Incidentally, the concentration of the enzymes measured in terms of maximum rate *in vitro* was only slightly greater than that required to furnish observed amounts of pyrimidines, and you do not need to have a drastic inhibition to throttle these reactions down.

Potter: The demonstration of the excretion into the medium of large amounts of material is of great importance. This proves the feedback; it is clear that this is not mass action, and that it is not effecting it by equilibrium but by direct inhibition of a prior enzyme. The fact that when uridine or cytidine is supplied, the excretion is completely shut off, is a powerful argument.

Pardee: As regards mass action, I should mention that one of these steps is irreversible according to Lieberman and co-workers (Lieberman, I., Kornberg, A., and Simms, E. S. (1955). *J. biol. Chem.*, **215**, 403). Therefore, there cannot be a mass action, a pushing back. In the second place, in mutants lacking an enzyme at any of a number of points, the mechanism still works.

Potter: Later on we can perhaps devote some time to a definition of what might legitimately be called feedback, and what is simply mass action or competition for substrate. (See p. 354).

Magasanik: In every case that has been studied so far, it is the very *last* product of a biosynthetic sequence, such as valine, isoleucine, threonine, histidine or cytidylic acid, which shuts off the *first* reaction of the sequence. This constitutes impressive evidence for the general importance of this control mechanism.

I would like to present to you how a complex system involving two cycles can be regulated in a simple manner by these feedback controls in *Escherichia coli* and related organisms. We have studied the terminal

steps in the synthesis of guanylic acid, the mechanism of the interconversion of purine nucleotides, and the rôle of these reactions in histidine biosynthesis. The reactions and the control points are shown in the following scheme:

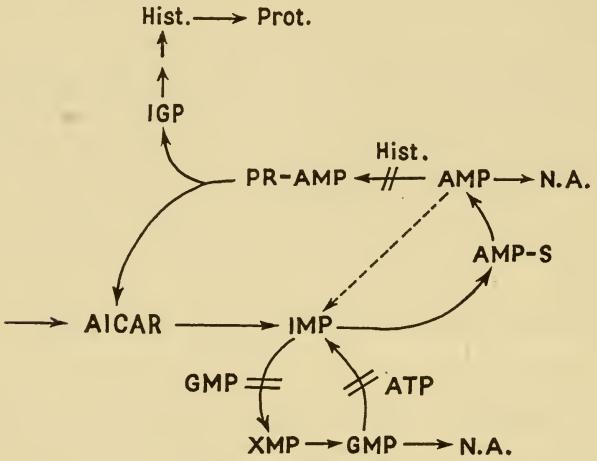

FIG. 1. (Magasanik).

It can easily be seen that IMP (inosine 5′-phosphate) occupies a central position in this scheme: it is converted to AMP (adenosine 5′-phosphate) via AMP–S (adenylosuccinate) (Lieberman, I., (1956). *J. biol. Chem.*, **223**, 327), and to GMP (guanosine 5′-phosphate) via XMP (xanthosine 5′-phosphate) (Magasanik, B., *et al.*, (1957). *J. biol. Chem.*, **226**, 339, 351). AMP and GMP produced in these reactions may be converted to di- and tri-phosphates which are essential participants in many enzymic reactions and building blocks for nucleic acids.

Each of the two reactions between IMP and GMP is irreversible; however, GMP can be converted back to IMP by still another irreversible reaction, catalysed by a TPNH-linked GMP-reductase (Mager, J., and Magasanik, B., (1958). *Fed. Proc.*, **17**, 267). The three enzymes responsible for these reactions together could catalyse the irreversible cyclic conversion of IMP via GMP to IMP, and would obtain the energy for the operation of this useless merry-go-round by the splitting of ATP, an essential part of the amination of XMP to GMP. This, however, does not happen. The reactions of the cycle are regulated in such a way that AMP and GMP are produced in just the amounts needed for metabolic reactions.

The conversion of IMP to GMP is controlled by GMP, which inhibits the action of the first of the three enzymes, IMP-dehydrogenase. Consequently a high intracellular level of GMP will prevent the formation of additional GMP from IMP, but will permit the conversion of GMP to

IMP, and thus the formation of AMP at the cost of GMP. This conversion is in turn controlled by ATP, which inhibits the action of the GMP-reductase; consequently, a high intracellular level of adenine nucleotides will prevent the formation of additional AMP from GMP. In addition, derivatives of the purines control synthesis of IMP *de novo* by inhibiting an early step of the common biosynthetic pathway (Gots, J. S. (1957). *J. biol. Chem.*, **228**, 57). The discovery of these control mechanisms explains why these organisms use exogenously supplied adenine or guanine preferentially to synthesizing these compounds *de novo*, and why, when supplied with a mixture of adenine and guanine, they use each purine preferentially for the production of the corresponding nucleotide component of the nucleic acids.

In addition to its other functions, AMP (or ATP) is also the donor of an N–C fragment which becomes the N_1–C_2 portion of the imidazole ring of histidine. The first step in the pathway leading to histidine is the attachment of ribose 5-phosphate to nitrogen-1 of AMP to give PR-AMP. This compound is aminated by glutamine and cleaved to give the histidine precursor IGP (imidazoleglycerol phosphate) and the purine precursor AICAR (4-amino 5-imidazole carboxamide ribonucleotide) (Moyed, H. S., and Magasanik, B. (1958). *J. Amer. chem. Soc.*, **79**, 4812). This series of reactions constitutes a cycle from IMP via AMP and AICAR back to IMP. The operation of this cycle is controlled by histidine, which inhibits the formation of PR-AMP from AMP (or ATP) and ribose 5-phosphate. The complete cycle will operate just sufficiently to supply the cell with the histidine it requires for the synthesis of protein; however, by blocking the path leading from AMP to AICAR, an excess of exogenously supplied histidine would also prevent the conversion of AMP to GMP, were it not for the existence of another, as yet unidentified enzyme, which catalyses the more direct formation of AMP to IMP. The function of this control mechanism could be demonstrated by experiments with [2-^{14}C]adenine. This compound was converted to guanine with considerably lower ^{14}C-content in a histidine-free medium, indicating considerable conversion via AICAR; in a histidine-containing medium, the guanine had the same radioactivity as the adenine, indicating the more direct conversion via IMP without loss of C_2.

It is hard to visualize how such a complex network of reactions could be controlled in a simpler fashion.

Racker: Can you elaborate on the point of differentiation between inhibition of enzyme activity and enzyme synthesis in reference to your own very interesting studies? Is the case of guanine, which inhibits enzyme synthesis as well as enzyme activity, an exception or a frequent occurrence?

Magasanik: In general, only the first enzyme of a particular biosynthetic sequence seems to be subject to inhibition by the ultimate product, while many of the enzymes of the sequence may be subjected to repression. In our laboratory, Mr. A. P. Levin has found that in *Salmonella typhimurium* guanine represses the formation of IMP-dehydrogenase (the enzyme responsible for the conversion of IMP to

XMP), as well as the formation of inosinicase (the enzyme responsible for the formation of IMP from its precursor, 4-formylamino 5-imidazole carboxamide ribotide). Only the former of these reactions, which is the first step of the branch pathway leading to GMP, is inhibited by GMP; the other reaction, which is a central step in the formation of both adenine and guanine, is inhibited neither by GMP nor by AMP, nor by any of their usual derivatives. Similarly, Dr. H. S. Moyed has found that the enzyme catalysing the first step of the pathway leading specifically to histidine, the condensation of AMP (or perhaps of ATP) with an activated derivative of ribose 5-phosphate, is both inhibited and repressed by histidine. The enzyme catalysing a subsequent step in histidine biosynthesis, the conversion of the AMP-ribose phosphate complex to imidazole glycerol phosphate, is repressed, but should not be inhibited.

I should add that Dr. Pardee has already made the point very clearly that the inhibition of enzyme action and the repression of enzyme formation do not have the same physiological function. The actual control of the biosynthesis of the precursors of macromolecules is achieved by inhibition of enzyme action; control by repression of enzyme formation is sluggish and would be effective only after several generations. Enzyme repression rather serves as a mechanism that enables the cell to select the most suitable enzymic composition for growth in a particular environment.

Lehninger: I had been wondering—and Dr. Magasanik has partly answered my query—why it would be strategically more desirable to have the end product of a long series come back and inhibit an early reaction.

Pardee: If the inhibition were half-way along the pathway, then some intermediate would accumulate in the pathway, presumably, and there would be the problem of getting rid of it; or at least the organism would waste that carbon.

Lehninger: Is there any other reason beyond the waste of intermediates that get stuck in the pathway? After all, there are branches here and there, and other metabolic pathways.

Pardee: In the threonine case which we have investigated lately (Wormser, E. H., and Pardee, A. B. (1959). *Arch. Biochem.*, in press), a pathway starts from aspartate and goes to homoserine, where it then branches and goes through perhaps two or three steps to threonine; the other branch goes off through another series of reactions to methionine. This was demonstrated (Watanabe, Y., and Shimura, K. (1955). *J. Biochem.* (*Japan*), **42**, 181) in yeast and confirmed by us in *Esch. coli.* We find that threonine inhibits homoserine kinase. The block of the threonine pathway is in the specific branch of the pathway that goes to threonine, and does not influence the methionine pathway.

Magasanik: You could continue that by saying that isoleucine acts at the point at which its pathway branches off from the threonine pathway.

Pardee: It has been shown earlier that isoleucine blocks an enzyme quite a few steps removed (Umbarger, H. E., and Brown, B. (1958).

J. biol. Chem., **233**, 415). Isoleucine inhibits, by feedback, the reaction of threonine deaminase which is on its pathway. Therefore, isoleucine could block its own synthesis without interfering with the synthesis of threonine or methionine.

Magasanik: The enzyme that converts threonine into ketobutyrate is quite irreversible, and so it is, therefore, quite advantageous for the cell to have the block at this point. A block after that would still be effective in preventing isoleucine from being formed, but it would permit a drain on the threonine, which would be converted to ketobutyrate and would thus not be available for protein synthesis.

Potter: There is a clear generalization emerging from the answers to Prof. Lehninger's question, i.e. that those mechanisms tend to survive in the course of evolution when the feedback block is effected at a branch point such that the inhibition of the synthesis of the product is specific, and interference with the synthesis of other products does not occur.

Lipmann: I should like to draw attention to the complication of building an enzyme of the type where there is not only a site for the substrate, but also one for the inhibitor, which seems to be specific and does not necessarily seem to be related to the substrate at all.

Magasanik: This touches on interesting observations made by Dr. Umbarger (Umbarger, H. E. (1957). *J. Bact.*, **73**, 105). He found that *Esch. coli* possesses two enzymes catalysing the same reaction: the conversion of threonine to ketobutyrate. One of these enzymes is produced by cells growing in a minimal glucose-containing medium. It plays an essential rôle in the biosynthesis of isoleucine, as shown by the fact that its loss through mutation results in a nutritional requirement for isoleucine. The action of the enzyme is inhibited by isoleucine. The other enzyme is produced in cells growing anaerobically in a medium rich in amino acids and free of glucose. It deaminates not only threonine, but also serine, and its metabolic rôle is presumably a catabolic one: it converts the amino acids threonine and serine to the keto acids ketobutyrate and pyruvate, which presumably are further metabolized to yield energy and building blocks for the formation of cell material. The action of this enzyme is not inhibited by isoleucine. Dr. Umbarger discovered a similar situation in *Aerobacter aerogenes* (1957, *J. Amer. chem. Soc.*, **79**, 2980). This organism contains two enzymes capable of converting pyruvate to acetolactate, a reaction essential for the biosynthesis of valine. One of these enzymes is formed by cells growing in a minimal medium at neutral or slightly alkaline pH, and is inhibited by valine. The other enzyme is only formed when the pH of the medium is low, and apparently is responsible for the formation of acetoin, a neutral product of glucose fermentation in place of acetic and formic acid; the physiological rôle of this enzyme seems to be the prevention of further production of acidity in the medium. The action of this enzyme is not inhibited by valine.

These two examples show that enzymes attacking the same substrates can differ strikingly in their affinity for inhibitors. Furthermore, it would appear that control of enzyme action by negative feedback is

of such importance for the survival of an organism that, in the course of evolution, selection will occur for the ability to form two enzymes catalysing the same reaction, but differing in their sensitivity to a controlling metabolite.

Racker: I am not sure that this answers Prof. Lipmann's question.

Lipmann: My question is difficult to answer, as has just been proved! It is not meant to be answered. I would only emphasize that the synthesis of an enzyme is complicated if you not only have to make a protein which has a site for a substrate, but also a site for the inhibitor.

Pardee: I wonder whether there are two sites. Perhaps the difficulty is more in the eye of the organic chemist than in the structure of the compound. In the case I mentioned, there is a competitive inhibition, and the simplest way of looking at it is that the substrate and the inhibitor compete with each other for a site.

Lipmann: But cytidylic acid and carbamyl phosphate are very different.

Pardee: They look very different, but perhaps we cannot see how they are shaped to be competitors.

Lipmann: There are many steps in between. Still, it has to be built for it in some way.

Pardee: Selection in bacteria is very fast. In the evolutionary process it takes only a matter of days for one mutant with a selective advantage to grow a population and reach the steady state in which it has overwhelmed the original type.

Potter: I disagree with an earlier comment made by Dr. Pardee. I think he was conservative when he extrapolated to animal tissue. He expressed the opinion that in animal cells, perhaps in contrast to bacterial cells, after the cells were formed the amounts of enzymes remained constant. I have been collecting literature on this subject. There are some very clearcut cases in which the amount of enzyme has changed dramatically in animal tissues over short periods of time under conditions which must involve feedback control, where new cells have not been formed.

Krebs: Have you in mind e.g. the cases reviewed by Knox and coworkers where enzymes are formed adaptively in animal tissues in response to dietary changes (Knox, W. E., Auerbach, V. H., and Lynn, E. C. C. (1956). *Physiol. Rev.*, **36**, 164)?

Potter: That is one example. An interesting case is that of Rosen and co-workers, on the glutamic-pyruvic transaminase induced by the administration of cortisone, dehydrocortisone or prednisone (Rosen, F., Roberts, N. Q., Budnick. L. E., and Nichol, C. A. (1958). *Science*, **127**, 287).

Krebs: The urea-forming system in the liver becomes much more active if one keeps rats on a protein-rich diet for a few days. In my introductory talk I mentioned the constancy of enzyme concentrations; the particular system I had in mind was the working muscle going from rest to activity; then the increase of metabolism is very great without, of course, a change in the amounts of enzymes. I quite agree that there are other circumstances where the amounts of enzymes increase.

AUTOMATIC ADJUSTMENT MECHANISMS IN BACTERIAL CELLS

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1. Introduction

Variations in the growth cycle

Unicellular organisms are inevitably exposed to changes of environment sometimes the result of the vicissitudes of their existence, sometimes the direct outcome of their own activity. In many ways they can undergo adjustments minimizing the adverse effects of the changed circumstances and tending to maintain the functioning of the cell as near as possible to an optimum. Such changes may have the appearance of being purposeful, but are in fact automatic and are brought about by the operation of what are in effect cybernetic mechanisms.

In a very common set of laboratory conditions a small number of cells are introduced into a medium of defined composition. They multiply, consuming nutrients, causing changes in pH, sending toxic products into the surroundings, and sometimes depleting the oxygen supply. Eventually growth stops, only to be renewed when some of the cells are again transferred to fresh medium. The sequence of events constitutes a growth cycle. Throughout this cycle continuous adjustments occur.

Changing concentration of nutrients demands, in fact, little compensation since the rate of growth is nearly independent of the concentration of substrate until this concentration falls to values in the region of 1 mg./l. The surface of the relevant enzymes is usually saturated with substrate at concentrations exceeding these very low values.

Changed pH, changed nature of the nutrients, transfer from

aerobic to anaerobic conditions, and the presence of toxic substances all demand active adjustments of one kind or another.

Even if the cells continued to grow in a medium maintained quite constant, adjustments would still be necessary. As the cell size increases, the ratio of area to volume diminishes. The ratio of chemical change per unit volume in the interior to rate of supply of nutrient and rate of removal of products therefore changes so that the metabolic balance is constantly disturbed. The changes are kept within relatively narrow limits by the periodic division of the cell, a process which itself constitutes an automatic adjustment ensuring continued efficient function.

When cells are transferred to quite new environmental conditions the necessary adjustments are similar to, though they may be considerably more drastic than, those coming into play during the normal growth cycle.

Kinetic principles of growth

The available cybernetic mechanisms are chemical in character, and depend upon the fundamental kinetic laws of growth. These have already been discussed on more than one occasion (Dean and Hinshelwood, 1955) and will be summarized only very briefly. Growth involves the reproduction of all cell parts. The synthesis and functioning of enzymes must therefore be intimately linked. The reaction pattern of the cell involves a complex set of consecutive and parallel reactions in which the products of one step are the reactants of the next. Cell division is triggered off by the formation of critical amounts of key substances. Simple and general mathematical treatment then shows that the proportions of the various cell constituents will adjust themselves gradually to such values that an optimum growth rate is established, and will tend to be maintained. If the conditions change, the enzyme proportions will change also.

The most important theorem here is that these changes take place in such a way as to keep the total growth rate at the highest possible value. An essential part of the kinetic scheme

on which it is based is the tendency for division to occur when a more or less standard amount of some key substance (or substances) has been formed.

Mutation

Another mode of adjustment relevant to populations rather than individual cells, and of significance in major changes to quite new environments rather than to the continuous variations accompanying the growth cycle, is that of mutation and selection. The commonly postulated preliminary to the operation of a selective mechanism is a discontinuous change in some structural element determining the heredity of the cell. Adjustments in individual cells on the one hand and selective population shifts on the other can of course be superposed. They are not mutually exclusive mechanisms.

If the change is rapidly and easily reversible it is normally ascribed to an adjustment in the individual cell activity, if irreversible it is commonly ascribed to a mutation followed by selection. But, as has been shown, this is not a significant distinction, since all degrees of reversibility are observed.

Types of response

In the first part of what follows, however, very easily reversible changes will first be considered. In section 2 an account will be given of the way in which certain enzyme activities vary during the growth cycle, and the significance of these variations will be considered in relation to the cell function. Section 3 deals with responses to changed conditions of aeration, and section 4 with the adjustments to changing pH. The part played by the division mechanism in regulating the cell economy is considered in section 5.

The discussion of these various adjustments leads on to the consideration of the way in which the cells respond to changes in their nutrient supply, and to the development of drug resistance. These topics involve a more detailed assessment

of the parts played by adaptive processes and by the mechanisms of mutation and selection.

2. Variations in Enzyme Activity during the Growth Cycle

In any normal growth cycle changes of pH occur and these cause variations in the amounts of certain enzymes according to a mechanism which is clear from the work of Gale and Epps (1942). The activity of unit mass of an enzyme is a function of the pH. If the pH is altered so as to lower the specific activity, more enzyme is required to effect the same amount of chemical change. Gale and Epps found that in certain cases as the pH became adverse the amount of enzyme per cell did in fact increase, the tendency being thus towards the preservation of a constant total intensity of chemical action. This result can be generally understood in the light of the kinetic principles, outlined in the introduction, which lead to the expectation of an optimum growth rate under the prevailing conditions.

The mechanism which operates in the experiments of Gale and Epps we may call simple pH response. That it is not the only mechanism causing variations in the amounts of enzymes in a cell during the growth cycle is clear from the work of McCarthy (1959), of which a brief summary will now be given.

McCarthy investigated the variations during a growth cycle of *Bacterium lactis aerogenes* of the amounts per cell of the following enzymes: catalase, glucose and succinic dehydrogenases, asparagine deaminase, and phosphatase.

The effect observed by Gale and Epps was found in a number of examples, especially with the deaminase. The amount rose steeply towards the end of the growth cycle as the pH fell. When an inoculum from the acid medium was transferred to fresh medium at pH 7 the amount of enzyme per cell rapidly fell, by a process of simple dilution (cf. Pollock, 1958), the new material synthesized at the more favourable pH containing a smaller content of the deaminase.

With catalase a more complex response to the fall in pH at the end of the cycle was observed. As the cells entered the stationary phase there was a very marked increase in the catalase content, far greater than would have been needed simply to compensate the lowered specific activity. If the pH fall was prevented by alkali addition the rise in catalase amount did not occur. The formation of the extra enzyme, however, appears here to be stimulated not directly by the adverse pH itself but by a factor (perhaps peroxide formation) which comes into being concomitantly with the pH change. This factor observably slows down growth, which now becomes limited, in the extreme case, by the rate at which the catalase system can be constituted to overcome the adverse circumstances. The enzyme activity per cell thus tends to rise steeply.

The behaviour of the dehydrogenases has some interesting aspects. Glucose dehydrogenase maintains itself at a nearly constant level throughout the growth cycle. Even when the cells have been grown with succinate instead of glucose as the sole source of carbon the glucose dehydrogenase still remains throughout at about its normal level. The strain of bacteria studied shows a very long lag on first being transferred to a succinate medium. During this lag there is, contrary to what might perhaps have been expected, no appreciable increase in the succinic dehydrogenase, which, however, increases very rapidly and markedly in amount as soon as actual growth starts. On the other hand, during the lag there is a considerable increase in deaminase activity. These observations show that the adaptation to succinate is a fairly complex process involving more than one enzyme. And indeed it is not altogether surprising that a new reaction pattern differing from the old in more than one respect has to be established. The succinic dehydrogenase does not reach its optimum value until several cycles in the succinate medium have been completed. On the return to the glucose medium of the succinate-adapted cells the succinic dehydrogenase falls to its original level, but not so rapidly as simple dilution of the

cell contents with the normal material of glucose-grown cells would require. Thus the succinic dehydrogenase system continues to be formed in excess of normal requirement for some time. Even when the succinic dehydrogenase has reverted to the level characteristic of growth in the glucose medium the cells have not yet returned to a state where they show a long lag for growth in succinate. This fact reinforces the conclusion that the enhanced succinic dehydrogenase activity is by no means the only factor concerned in the adaptation to the succinate medium.

The phosphatase activity is characterized by one rather remarkable property, namely rapid increase to pronounced maximum in the early stages of growth, and subsequently rapid decline at a rate which suggests "dilution-out" by the formation of material containing much less of the enzyme. Neither the early rise nor the later fall is due to changes in pH. The initial burst of phosphatase activity seems to occur in direct response to the needs of the young cells at the start of the growth cycle.

3. Aerobic-Anaerobic Transitions

One kind of environmental change to which cells are not infrequently exposed and which calls for an appropriate regulation of the metabolism is the transfer from aerobic to anaerobic conditions and *vice versa*. The behaviour of *Bact. lactis aerogenes* when confronted with these needs for physiological adjustment is typical of cell mechanisms in general, though the behaviour of other bacteria may well differ a good deal in detail.

Cultures of this organism show a reducing power towards oxygen which can be given a quantitative measure by tests involving the consumption of oxygen and the reduction of methylene blue. Let the measure of this reducing activity be denoted by R. For a wide variety of carbon substrates R attains a constant limiting value, which is the same from substrate to substrate when once the cells have become fully

adapted to aerobic growth on the one in use. The different carbon sources, however, support growth at very different rates, from which it may be inferred that the growth of the fully adapted culture is limited in rate by a part of the cell mechanism which consumes molecular oxygen. When the cells are first offered one of these substrates, however, growth is much slower than the optimum and R is correspondingly less than the maximum value. As adaptation to the new source proceeds R and the growth rate increase in a parallel manner to their respective optima. It seems, therefore, that until the enzyme proportions have settled down to equilibrium values the substrate itself can not be metabolized up to the limit permitted by the mechanisms consuming molecular oxygen. During aerobic growth in a new carbon substrate, then, the first adjustment is a slow establishment of enzyme ratios allowing the full utilization of the oxygen supply (Baskett and Hinshelwood, 1950).

If an adapted aerobic culture is made anaerobic while in active growth, multiplication is at once arrested and the value of R rises. After a considerable but variable delay growth is resumed at the lower rate corresponding to the anaerobic conditions, R drops more or less to its original value and the consumption of the carbon source for each unit of bacterial mass produced becomes much larger. These adjustments do not occur in the absence of a nitrogen source. It appears that the resumption of growth in the anaerobic conditions demands the synthesis of new enzyme facilities, permitting the reoxidation of reduced carriers no longer by molecular oxygen but, directly or indirectly, by the carbon substrate. Until these new facilities have been developed by adaptive adjustments, reduced carriers accumulate in the cell and may account for the enhanced value of R observed during the arrest period.

When, on the other hand, a culture growing anaerobically is suddenly provided with aeration the response is almost immediate and the growth rate rises without any period of delay to the higher value. The consumption of the carbon substrate rapidly falls to that characteristic of aerobic

metabolism. No enzyme adjustments appear to be necessary, the cells having apparently retained unimpaired their ability to utilize molecular oxygen when it becomes available (Baskett and Hinshelwood, 1951*a*).

The increased consumption of carbon source in anaerobic growth, and the increased economy of utilization in presence of oxygen is, of course, an example of the well known Pasteur effect. The part of this phenomenon which at first sight presents an almost purposeful air is the inhibition of the wasteful substrate consumption as soon as oxygen is provided to give a more efficient mode of utilization. The mechanism is, however, purely automatic, depending essentially on the fact that oxygen immediately lowers the ratio of the reduced to the oxidized carriers in the system and thereby largely cuts out the competing reactions into which the former might have entered. The saving of substrate depends upon the simple chemical fact that whereas a carbon compound can be oxidized fairly readily to carbon dioxide and water, it can only act as a hydrogen acceptor in the fermentative type of reaction relatively inefficiently since the formation of fully reduced products such as paraffins is structurally seldom possible (Baskett and Hinshelwood, 1951*b*).

4. Alkali Metal Ions and pH Regulation

The passage of alkali metal ions into or out of the cell plays a major part in regulating carbohydrate metabolism. It also plays some part, in our view an indirect one, in the response of the cell to adverse pH changes, a phenomenon in which other factors are also important. The more general aspect of alkali metal ion metabolism will therefore be considered first (Eddy and Hinshelwood, 1951; Eddy *et al.*, 1951).

With *Bact. lactis aerogenes* K^+ or Rb^+ ions are necessary for growth, and cannot be replaced by other members of the alkali metal series (Eddy and Hinshelwood, 1950). Smaller as well as larger ions are ineffectual, so that a specifically

correct ionic radius is evidently more important than mere permeability, a conclusion borne out by the work of Roberts and his colleagues (Roberts, Roberts and Cowie, 1949; Cowie, Roberts and Roberts, 1949; Roberts and Roberts, 1950).

During metabolism a positive stream of alkali cations enters the cell together with the negative stream of phosphate ions. The alkali cations appear to become incorporated in some structure involving an enzyme surface where phosphorylated intermediates of the carbohydrate metabolism are dealt with. This specific structure appears important in explaining the size specificity, and its stability can be invoked to explain the action of the alkali ions in displacing hydrions. In this sense the potassium or rubidium ions protect the cell against an adverse pH. If the hydrion concentration of the cell rises, enzyme reactions by which phosphate is incorporated into the cells are slowed down. The alkali ion which would normally be released to deal with more phosphate remains bound and the total amount of K^+ or Rb^+ in the cell rises. Observations with radioactive tracers show that when the pH of cells actively metabolizing glucose is lowered there is a temporary displacement of alkali ion, due to the direct competition of hydrion, followed by an increase in the maximum uptake as the internal adjustments are completed. When the carbohydrate metabolism ceases the alkali metal ions leave the cell. From resting cells, decrease in pH facilitates this displacement. The alkali ion is not an essential structural component of the cell, at any rate in considerable amounts, but is an enzyme activator and metabolic regulator.

If dehydrogenase activity is adversely affected by increasing acidity, addition of potassium ions lessens the fall, an antagonistic action not in evidence with deaminase activity.

The influence of pH on the growth rate of the cells is interesting. Over a wide range, pH 7 to 5, the actual value of the initial generation time is unchanged, though the total population which the medium can support falls steeply as the acidity increases (Lodge and Hinshelwood, 1939). If the pH of a dividing culture is suddenly dropped by about 0·7 unit

there is a retardation of growth followed by a recovery which indicates some active adjustment of the cells. This response fails if the medium is much more acid than pH 6 or if its acidity changes too rapidly, as occurs when the buffer breaks towards the end of growth in a concentrated glucose medium (Eddy and Hinshelwood, 1951).

5. Cell Division

Division of the cell may be advanced or delayed in relation to other synthetic processes, and since those themselves vary in rate earlier or later cell division may influence the ratio of the constituents.

Determination of the nucleic acid content of cells reveals one major tendency, namely for the content of deoxyribonucleic acid (DNA) to remain nearly constant in spite of changes of conditions which considerably influence cell size and growth rate (Boivin, Vendrely and Vendrely, 1948; Mirsky and Ris, 1949; Caldwell and Hinshelwood, 1950). This tendency may be strained too far and break down in extreme conditions, but is clearly the reflexion of one major regulating mechanism. There is on the other hand a much greater variability in the ribonucleic acid (RNA) content, and a general tendency is observable for the RNA to increase in amount in conditions giving enhanced growth rate (Caspersson, 1947; Malmgren and Héden, 1947; Caldwell, Mackor and Hinshelwood, 1950).

The information stored in the DNA is recognized to be a major factor determining heredity. Since, however, the cell is probably a hierarchial organization, cytoplasmic structures above the level of DNA codes may also have considerable influence on the properties of cells formed by binary fission, and play a part in determining the persistence over several generations of biochemical characters impressed by adaptive processes.

There is a close linkage between the synthesis of nucleic acids and that of proteins, and a good deal of inconclusive

controversy has centred round the mode of coupling. The uncompromising attribution of autosynthetic properties to nucleic acid alone seems an improbable oversimplification. On general grounds a complex mutual interdependence of all the synthetic processes seems much more likely.

A model for the DNA molecule and a scheme for its replication have been suggested by Watson and Crick (1953). They claim that the DNA molecule in the cell is composed of two coiled chains held together by hydrogen bonds between the bases, a single base from one chain being hydrogen bonded to a single base from the other. From evidence which will not be detailed here they conclude that the only possible pairs of bases are adenine with thymine and guanine with cytosine. The phosphate-sugar backbone of the model is completely regular but any sequence of the pairs of bases can fit into the structure. In a long molecule, therefore, many different permutations are possible, and according to Watson and Crick it seems likely that the precise sequence of the bases is the code which carries the genetical information. Moreover, if the actual order of the bases on one of the chains were given, one could write down the exact order of the bases on the other, because of the specific pairing. In other words, one chain is the complement of the other. According to this scheme, duplication of the DNA molecule involves the breakage of the hydrogen bonds and the unwinding and separation of the two chains. Each chain then acts as a template for the formation on to itself of a new companion chain.

In the chromosomes of *Crepis capillaris*, however, Plaut and Mazia (1956) find that the parental DNA is not equally distributed between the products of chromosomal duplication as the Watson and Crick scheme would predict, and suggest that it is necessary to find an alternative mechanism for DNA duplication which would eliminate the permanent separation of the two strands of the double helix, or to assume that only part of the DNA is involved in the reproductive process. Arley (1955) finds difficulty in the Watson-Crick scheme for DNA replication in that the energy required to break the

hydrogen bonds joining the parallel helices would be considerable, and maintains that a similar criticism would apply to the mechanism of Delbrück (1954) whereby the chains are separated and later recombined by a series of breaks and reunions in a zipper-like fashion. Arley states that Gamow's suggestion (1954) of a one to one correspondence between the double polynucleotide chain of a DNA molecule of the Watson-Crick type and the single polypeptide chain of a protein (or enzyme) molecule of the Pauling and Corey (1951*a* and *b*) type, leads to a simpler mechanism. In it the linear information contained in the DNA molecule is translated one to one into that of a protein molecule, each of the possible (about 20) quadruples of purines and pyrimidines corresponding to one of the possible (about 20) amino acids. Since the correspondence is one to one Gamow has shown that a protein (or enzyme) molecule can be built up "on the inside" of the double helix of a DNA molecule so that the order of the amino acids is uniquely determined by the order of the purine-pyrimidines. This new protein molecule can now serve as a template for the building up of a DNA molecule "around" the protein molecule as axis so that the order of the purine-pyrimidines is now uniquely determined by the order of the amino acids.

Another model involving the participation of protein in DNA replication has been proposed by Butler (1956). In it the twin fibre of DNA is closed at one end and the single fibres are attached to histone at the other end. A pull on the histone fibres will separate the two strands of the DNA (zipper fashion) and at the same time uncoil the spiral. It is supposed that the DNA fibre replicates in the open position and that another pull on the histone fibres in the middle will cause each to close up. If this is correct each new DNA particle formed will be made of newly synthesized material.

Whichever of these theories is correct it seems likely that proteins and nucleic acids are intimately interconnected in the cell and the evidence that nucleic acid is concerned in protein synthesis has recently been reviewed by Gale (1958).

Application of the cytochemical methods of Holt and his collaborators (Holt and O'Sullivan, 1958) has shown strikingly that during mitosis enzymes normally having a peripheral distribution in the rat liver cell gather into the nuclear region. One very suggestive view of this phenomenon is that they are called upon in some way to exchange information with the nuclear elements themselves.

Conclusive evidence about the protein/RNA/DNA interdependence is still lacking. In the meantime, several lines of investigation are yielding useful evidence. On the one hand, cells may be broken down by various means, separated (e.g. by centrifugation) into particulate ranges of different sizes, and the residual biochemical properties of these can be investigated. On the other hand, normal growth of intact cells may be disturbed in various ways, by temperature changes, by the addition of drugs, or the withholding of growth requisites, and the consequent changes in function, and in nucleic acid or protein content observed. Among examples of such investigations may be cited work on the inhibition of protein synthesis by chloramphenicol (Gale, 1958); Cohen's work on the unbalanced growth caused by incubating a thymine-requiring mutant in a medium lacking thymine whereby DNA synthesis is held up while certain other growth processes continue (Barner and Cohen, 1954; Cohen, 1957); and work on synchronization, whereby it appears that the cells can be brought into a state where they are ready in most respects for division except for the formation of sufficient DNA, so that when the synthesis of this can go forward, all divide together. Some of the details of this work are of special interest in connexion with the general problem of regulation.

The action of chloramphenicol on protein synthesis and on nucleic acids has recently been fully reviewed by Gale (1958) and need not be repeated here in detail. Of particular interest to the present discussion is the work of Pardee, Paigen and Prestidge (1957), Neidhardt and Gros (1957), Hahn and coworkers (1957), Ben-Ishai (1957) and Barner and Cohen (1957).

Pardee, Paigen and Prestidge have shown that the RNA of *Escherichia coli* can be separated by electrophoresis into two fractions. The major fraction has a high molecular weight and is firmly bound to protein. The other fraction has a molecular weight of less than 5000 and a higher electrophoretic mobility, apparently a different base composition, and seems to be less firmly bound to protein.

The RNA content of cells in which the synthesis of protein is inhibited by chloramphenicol, increases on incubation and the new RNA is similar in electrophoretic mobility and firmness of binding to the low molecular weight component of Pardee, Paigen and Prestidge. In base composition, however, it resembles the major fraction and seems to differ from the normal minor component in size. Neidhardt and Gros (1957) have shown that the RNA formed in the presence of chloramphenicol and in the absence of concomitant protein synthesis in *Esch. coli* is extremely unstable and breaks down when the cells are subsequently incubated in the absence of the drug. The resulting fragments, however, are resynthesized into RNA when chloramphenicol and a source of energy are added. In a similar sort of study Hahn and co-workers (1957) find that cells of *Esch. coli* B/r, whose protein synthesis is inhibited by chloramphenicol, accumulate several times the amount of intracellular nucleic acids contained in log phase cells before the onset of chloramphenicol-induced bacteriostasis. On removal of the drug, the bacteria recover and much of the excess of nucleic acids is ejected before growth and multiplication are resumed. It seems that an imbalanced synthesis of normal nucleic acids takes place under the influence of chloramphenicol rather than the formation and elimination of abnormal polynucleotides.

That RNA preformed in the absence of protein synthesis participates neither in subsequent protein synthesis nor in induced enzyme (β-galactosidase) formation was concluded by Ben-Ishai (1957).

It is generally believed that DNA is a more stable component of the cell than RNA. In the experiments of Neidhardt

and Gros (1957), although chloramphenicol treatment increased the RNA content of the cells by about 70 per cent it also increased the DNA content by 50 per cent. Unlike the additional RNA, however, the DNA was stable in the absence of the drug. Barner and Cohen (1957) have managed to double the DNA content of a bacterial mutant unable to synthesize thymine and phenylalanine by preincubation in a minimal medium containing phenylalanine followed by incubation in the presence of thymine alone. They conclude that the preincubation with the amino acid may stimulate the formation of a protein possibly essential to the subsequent synthesis of DNA.

In what has been cited above the controls are in a sense imposed on the cell from without, but analogous mechanisms will be involved in the kinds of control a cell can exercise on its own processes.

In the work described in section 2 the variations during the cellular growth cycle of certain enzymes have been referred to. Variations of the RNA and DNA during growth cycles also occur, and the detailed investigation of these and their relations to the enzymic changes will undoubtedly prove to be of great interest.

6. Adaptive Phenomena

Reversible adjustments in the quantitative relations of the cell functions have been evident throughout the foregoing discussion. Sometimes the establishment of a steady state is rapid and is nearly complete as soon as the newly formed cell material outweighs the old. Sometimes, however, the change is sluggish, and physicochemical explanations of the slow reversibility can, in fact, be offered (Dean and Hinshelwood, 1955).

A slowly reversible change in the enzymic composition of the cell would constitute a natural explanation of resistance induced by drugs, and of the emergence in presence of substrates such as lactose or D-arabinose of bacterial strains which

have become able to utilize them after an initial reluctance to do so. The difficult reversibility of such adaptations has often been used as an argument to support the view that the new forms are genetically different, containing presumably DNA patterns modified by accidental mutation. But the variable speed of reversion of cell adjustments in general seems to invalidate at least this particular form of argument. A mutant may indeed be more resistant than the original form, but more proof than slowness to lose the resistance is needed to establish that it is due to mutation rather than to an adjustment induced by the drug.

Our views on this question were summarized in a communication to a previous symposium of the Ciba Foundation (Dean and Hinshelwood, 1957*a*), and will not be repeated here. They in no way under-rate the significance of information stored in DNA patterns, but they allow considerable scope for quantitative modification of cell characters by enzymic adjustments, some of which may be established quickly in new conditions, and reversed slowly on return to the old conditions.

Two developments will, however, be referred to here in extension of what was said previously. Evidence cited in favour of the view that there is a formation by direct adaptation of D-arabinose-positive and lactose-positive forms of coliform bacteria from the original "negative" strains was called in question at the symposium itself. The basis of the objections was the claim that during the sojourn of the bacteria in the medium where the adjustment is taking place platings of samples on to solid media containing D-arabinose on lactose gave two types of colony clearly recognizable as positive and negative. The answer to this objection, in our view, is as follows. The adaptation times of individual cells vary according to an approximately normal distribution. Qualitative visual observation of a set of colonies with diameters distributed in this way can be misleading, and easily give the impression of "large" and "small" types, whereas quantitative measurement shows a continuous dis-

tribution. In a repetition of previous experiments we have found only continuous distributions of sizes on plates seeded with samples taken from lagging cultures before growth begins. After growth has actually started, bimodal distributions may sometimes appear. The reason for them, we believe, is that adaptation, although it has progressed some way, is demonstrably by no means complete at the moment when division and growth become possible, but after active growth sets in adaptation rapidly proceeds further. Thus, the cells which have already started to grow may for a time run far ahead of those which have not yet begun. Hence the temporary separation of the culture into two types: cells which have not yet undergone division and multiplication, on the one hand, and on the other those which have.

Arguments about mutants have also been based on the formation of papillae on colonies. But we have argued (Dean and Hinshelwood, 1957*b*) that the problem of papilla formation is much the same whether the new growth consists of mutants or of adapted cells.

The fundamental problem whether adjustments occur at the level of the individual cell, or by a shift among components of a population, can be most directly answered by the observation of the individuals themselves. In this connexion we should like to offer a comment on the beautiful experiments of Hughes.

The process of response to penicillin and chloramphenicol is strikingly shown in the microfilms taken according to the technique of Hughes (1957).* A few cells are seen in medium containing the inhibitor at a concentration so chosen as to slow down growth without inhibiting it entirely. In the presence of penicillin the majority of them are seen to grow, often with the production of elongated or abnormal forms. Survivors from this first culture are taken and subjected to slightly higher concentrations. In general, about half the

* Film shown at the Ciba Foundation Symposium on Drug Resistance in Micro-organisms; obtainable from the Photographic Department, St. Mary's Hospital, London.

yield of the first batch prove viable in the second. The cycle is repeated with gradual increase in concentration and eventually a highly resistant strain is obtained. One very interesting run of film shown by Hughes presents in parallel two small groups of cells, one (A) subjected to the action of chloramphenicol, and the other (B) not. The successive patterns observed in the two cases follow a sequence as nearly as may be seen identical, except that (A) appears as a slowed-down version of (B).

The processes made visible in these records are not those suggested by theories ascribing the development of resistance solely to the selection of rare mutants. All the cells visible on the film at a given moment appear to be growing, although more slowly in presence of the drug than in its absence. They are clearly performing sufficient of their total complement of biochemical reactions to achieve material synthesis against the action of the inhibitor. After each cycle in the series of transfers the yield of cells which can be transferred to a higher concentration with prospects of survival is as much as one in two. From these observations, therefore, it seems clear to us that active adjustments in the cell economy occur.

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DISCUSSION

Dean: In the cell there is a tendency for the DNA content to remain nearly constant under a wide variety of experimental conditions in which the growth rate varies considerably, although in extreme cases

the constancy may break down. On the other hand, the RNA content tends to be proportional to the growth rate, but here again the tendency may break down in extreme cases.

We have been interested in following the change in the RNA/DNA ratio in cells of *Bact. lactis aerogenes* (*Aerobacter aerogenes*) during the

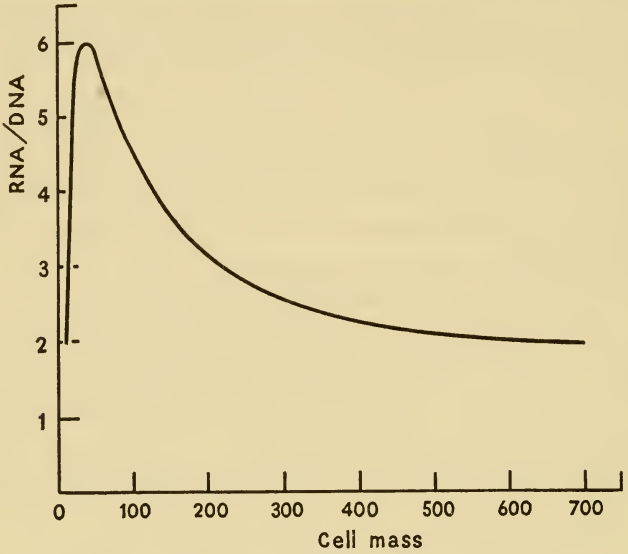

FIG. 1. The variation in the RNA/DNA ratio during a growth cycle.
Ordinate: RNA/DNA
Abscissa: cell mass.
The cell mass was determined turbidimetrically and is expressed in terms of the number of cells of standard size in millions/ml.

growth cycle in a simple synthetic medium containing glucose, phosphate buffer (pH 7·1) and magnesium and ammonium sulphates. The general pattern which emerges is shown in Fig. 1.

In these experiments the inoculum was taken from a fully grown culture of an earlier growth cycle and would consist of cells whose RNA/DNA ratio was about 2. Reference to Fig. 1 will show that there is a considerable rise in the RNA/DNA ratio in the early part of the cycle followed by a slower fall as growth proceeds, the ratio eventually settling down to a value of about 2. The curve was not greatly different when the air supply to the culture was replaced by nitrogen. In the normal course of one of these growth cycles the pH of the culture medium gradually changes from 7·1 to about 4. Keeping the pH

constant at 7·1 by the graded addition of alkali had little effect on the result.

What is of particular interest to us at present is the stimulus for the sudden increase in the RNA/DNA ratio in the early part of the growth cycle but so far we have no information. The addition of amino acids such as asparagine and glutamic acid did not prevent the early rise. During the early part of the growth cycle there is a considerable increase in cell size. For instance, by the time the RNA/DNA ratio has reached its maximum value the cell mass has increased fivefold but the actual number of cells is usually twice the number inoculated. Thereafter, the cell size diminishes throughout the growth cycle.

The falling part of the curve, on the other hand, can be explained by the progressive dilution out of material having an RNA/DNA ratio of 6 by material with the ratio of 2 reached at the end of the growth cycle.

Lipmann: Dr. Dean, would you comment on the absolute amounts of DNA and RNA during this cycle?

Dean: The DNA per unit of mass tends to keep constant, whereas the RNA per unit of mass tends to fall; so the change is taking place in the RNA more than in the DNA of each cell.

Lipmann: Then there is an initial formation of RNA and dilution of the RNA as it is formed, and it is not apparently formed after that?

Dean: We are diluting out the material with the high RNA content with material having a low standard amount of RNA.

Magasanik: Regarding the observation by Dr. Dean and Sir Cyril that the phosphatase seems to increase in the early part, I think there is some evidence that, at the beginning of glucose fermentation, there is a drop in the intracellular phosphate.

Hess: Only in the ascites cells, to my knowledge.

Magasanik: Dr. Torriani studied the formation of phosphatase in *Esch. coli*, and found that the alkaline phosphatase was subject to repression, i.e. in cells growing on a phosphate-containing medium this enzyme was present in small amounts. It was shown by Levinthal and Garen that cells grown without inorganic phosphate formed the enzyme in fabulous amounts, up to 5 per cent of the total weight of the cell. It seems that inorganic phosphate, as Dr Pardee has described previously, repressed the formation of phosphatase, which is an enzyme responsible for the production of phosphate. I wonder whether the observation by Dr. Dean and Sir Cyril that the phosphatase first increases and then decreases again, might not reflect the level of inorganic phosphate in the cell at the beginning of growth; when the level of phosphate inside the cell drops, one would expect a rapid synthesis of phosphatase which would be followed, after a high level of phosphate has been reached, by the inhibition of further synthesis of the enzyme.

Hinshelwood: The cells are all the time in a medium containing plenty of phosphate.

Magasanik: In the ascites tumour cells in a phosphate-containing medium, we have seen that the intracellular level drops when glucose metabolism begins.

Hinshelwood: We wondered whether phosphatase perhaps plays a rôle

in the synthesis of RNA and whether the rise in RNA and the increase in phosphatase content might not be closely connected. The two curves are not the same, but they are similar.

Magasanik: Levinthal's results would not support this idea: he obtained mutants which could not form phosphatase, and which grow very well in a medium containing inorganic phosphate, but had lost the ability to grow in media containing an organic phosphate compound as the sole source of phosphate. Apparently this particular phosphatase is not essential for growth.

Hinshelwood: The conditions of formation of the two might be similar.

Lehninger: This discussion reminds me of our discussion yesterday about the requirement of inorganic phosphate to get things going. Like many enzyme chemists, I have often wondered about the rôle of alkaline and acid phosphatase in cells. Dr. Racker (1955, *Physiol. Rev.*, **35**, 1) made the interesting suggestion that they are possibly artifacts; that they may be expressions of the activity of some polyfunctional enzyme, like triose phosphate dehydrogenase which we know can split acetyl phosphate, for example. In this connexion, can he say whether this enzyme has ever shown the ability to split 1 : 3-diphosphoglycerate, because it might be possible to find an explanation of what phosphatase activity is doing in the cell.

Racker: We are quite certain of the acylase activity of triose phosphate dehydrogenase in respect to acetyl phosphate. We also know that the acyl enzyme formed with 1 : 3-diphosphoglycerate is quite unstable in the presence of DPN. But I would hesitate to give an accurate evaluation of the rate of cleavage of 1 : 3-diphosphoglycerate by the enzyme, because of the rather rapid spontaneous hydrolysis of this compound. I like the suggestion that phosphatases may serve as regulators of intracellular phosphate concentration and I had something similar in mind earlier when I discussed the possible rôle of ATPase in allowing the Krebs cycle to go on under conditions of ATP over-production (see p. 215). Dr. Magasanik, do the phosphatase-deficient mutants require relatively high concentrations of inorganic phosphate for growth?

Magasanik: They require the usual amount. If the inorganic phosphate is replaced by glycerol phosphate, then the mutant cannot grow. If there is more than one phosphatase, then this one seems to be the important one.

Potter: That is the important point: there is more than one phosphatase. A discussion of phosphatase is one of the most embarrassing subjects for a biochemist, and until we know what phosphatases we are discussing, the discussion is not likely to be very useful. It may turn out that some of the enzyme activity which is called phosphatase may have something to do with protein synthesis. We must wait until the current studies on protein synthesis are correlated with phosphatase activity. I am sure that the studies to which Dr. Magasanik referred do not result in the production of cells which have no phosphatase activity.

Magasanik: Levinthal's results indicate that unless the cells have this particular phosphatase, they do not have either the right enzyme or enough enzyme to be able to produce sufficient inorganic phosphate

from glycerol phosphate to grow. They may have other phosphatases which are not sufficient in quantity or not active under these conditions and may serve other purposes.

Gutfreund: May we assume for the moment that what Sir Cyril and you are talking about is what is known as alkaline phosphatase?

Magasanik: Yes.

Hinshelwood: No, we used glycerol phosphate for one substrate and phenolpthalein phosphate for the other, and both gave the same cycle.

Gutfreund: Did you try pH-dependence of the enzyme?

Hinshelwood: Yes, it was used very near to the optimum. I think it was slightly alkaline.

King: Sir Cyril mentioned that the catalase increases where the process slows down. There is a micrococcus which is resistant to very high radiation dosage, and this micrococcus has many times more catalase content than the ordinary micrococcus. Dr. Laser is studying this at the Molteno Institute.

Chance: I have made a similar observation on the amount and activity of cytochrome a_3 of log and stationary phase Torula suspensions. Early log phase cells grown in a "chemostat" showed a respiratory enzyme content rather low in relation to respiratory activity. The turnover number of cytochrome oxidase was the highest that we ever observed. There appears to be least oxidant capacity when it is most needed. This is, however, compensated by the metabolic control which allows an unusually high turnover number of the available enzyme.

Siekevitz: Dr. Dean, have you done experiments to determine the turnover rate of RNA during that growth cycle?

Dean: No.

Siekevitz: Is it broken down once it is formed in the cell?

Dean: Towards the end of the growth cycle, when conditions are becoming unfavourable, there is a tendency for the DNA to be kept constant; if conditions are adverse, a transformation from RNA to DNA may occur, in an attempt to keep the DNA constant. DNA seems to be synthesized from RNA in conditions of phosphorus starvation.

Krebs: Dr. King, is it thought that the high catalase content of this micrococcus you mentioned is directly connected with lack of radiosensitivity? One could test it experimentally by adding an inhibitor of catalase, such as hydroxylamine, and examining the sensitivity to ionizing radiations of the treated organism. Has that been done?

King: No, this organism was first isolated in Oregon from a classified project.

REGULATION OF GROWTH AND COMPOSITION OF THE BACTERIAL CELL

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During the past decade most of the enzymic reactions responsible for the biosynthesis of amino acids, purines and pyrimidines have been elucidated. It has now become possible to ask by what mechanisms the many individual pathways of biosynthesis are co-ordinated with one another and with the energy-producing reactions of the cell; for it is obvious that growth, the orderly synthesis of protoplasm, requires a highly effective integration of the anabolic and catabolic activities of the cell.

Two recently discovered control mechanisms have been discussed in a preceding paper (Pardee, this symposium, p. 295): they are the inhibitory effects exerted by the ultimate product of a biosynthetic sequence (an amino acid or a purine- or pyrimidine-nucleotide), (1) on the action, and (2) on the formation of an enzyme catalysing an earlier step of this sequence. It is the first of these mechanisms which is presumably responsible for continually adjusting the rate of synthesis of the metabolic intermediates according to the demands of protein and nucleic acid synthesis; the second mechanism would be too sluggish for the effective control of this process.

The significance of enzyme repression [a convenient name coined recently by Vogel (1957*a*) for the specific inhibition of the formation of an enzyme, usually by the ultimate product of its action] becomes clear when we consider the ability of micro-organisms to adapt themselves rapidly to great changes

in their environment. For example, *Aerobacter aerogenes*, the organism used in our experiments, can grow readily in media containing glucose, glycerol, *myo*-inositol, histidine, or any one of many other compounds as the sole source of carbon and energy. This versatility of micro-organisms depends on their ability to convert compounds of different structure by means of specific enzymes to the same essential metabolites; however, the total capacity of the cell for protein synthesis is limited, and the larger the number of different enzymes which the cell contains, the smaller will be the amount of each individual enzyme. Repression of the formation of an enzyme by a product of its action enables the cell to dispense with making more of the enzyme than is needed to provide the product at a level sufficient for optimal growth; consequently, the cell can use its capacity for protein synthesis advantageously to produce more of those enzymes whose products are in short supply.

It has long been known that the rate of formation of certain enzymes is enormously increased when their substrates are added to the growth medium. In general, enzyme induction seems to have the same physiological significance as enzyme repression: both mechanisms select, within the genetical framework of the cell, the enzymic composition best suited for growth in the particular environment. It is therefore pertinent to consider the mutual relationship of these two regulatory mechanisms.

The constitutive repressible enzymes catalyse essential steps in the biosynthesis of amino acids, purines and pyrimidines. These enzymes are readily produced by mutants with genetical blocks prior to the step catalysed by the repressible enzyme (Vogel, 1957*b*; Gorini and Maas, 1957; Yates and Pardee, 1957). The substrate of the repressible enzyme is neither produced by these mutants nor provided in their growth medium, and it would therefore appear that its presence is not required for the formation of the enzyme.

On the other hand, the study of the inhibitory effect exerted by glucose on the formation of many inducible enzymes, has

led us to conclude that this effect is a manifestation of enzyme repression (Neidhardt and Magasanik, 1956*a*, 1957; Magasanik, 1957; Magasanik, Neidhardt and Levin, 1958). Glucose is metabolized much more rapidly than other carbon compounds and it was postulated that the products of its degradation are formed faster than they can be removed by the anabolic reactions of the cell. Glucose inhibits the formation of an enzyme only when the ultimate product of the action of the enzyme is a metabolic intermediate which is also readily produced by the degradation of glucose. The congruity of this phenomenon with that of enzyme repression is clear: the formation of the enzyme is inhibited by the ultimate product of its action.

This concept of the "glucose effect" is supported by the results of an investigation of glucose metabolism in *A. aerogenes*.* During growth in a medium containing mineral salts, ammonium sulphate and glucose, the organism excretes a variety of degradation products of glucose, including pyruvate, gluconate, and 2-ketogluconate, into the culture fluid; after exhaustion of the glucose the cells continue to grow on these degradation products at a diminished rate. Cells harvested during exponential growth on glucose and suspended in phosphate buffer, oxidize glucose and gluconic acid rapidly, but oxidize pyruvate very slowly; cells harvested after they have exhausted the glucose and entered the stationary phase, oxidize all three substrates rapidly. Cells grown on glycerol, whether harvested during or after the exponential phase of growth, oxidize glucose and pyruvate rapidly, but oxidize gluconate slowly (Table I). Glucose-grown cells, suspended in the mineral salts medium from which ammonium sulphate has been omitted, oxidize glucose much more rapidly than when suspended in phosphate buffer; this stimulation of glucose metabolism was found to be due to the Mg^{2+} which the medium contains. Interestingly

* The experimental work described in this section was carried out by one of us (A.K.M.) at the Children's Cancer Research Foundation and was supported by U.S. Public Health Service Grants C–1691 and CY–3335.

enough, the rate of glucose oxidation by glycerol-grown cells was not enhanced by Mg^{2+}. The effect of Mg^{2+} on the glucose metabolism of glucose-grown cells consists in the stimulation

Table I

RATES OF OXIDATION OF GLUCOSE AND OF ITS DEGRADATION PRODUCTS IN *Aerobacter aerogenes*

Growth medium ...	*Glucose*		*Glycerol*	
Growth phase ...	*Expt.*	*Stat.*	*Expt.*	*Stat.*
Substrate				
Glucose	53	56	68	53
Gluconate	67	62	18	17
Pyruvate	15	54	58	53

The rates are expressed as μl. of O_2 taken up in one hour by one unit of cells in phosphate buffer at pH 6·4 and 37° (Magasanik, Brooke and Karibian, 1953).

of the production of gluconate, pyruvate and α-ketoglutarate from glucose, and of the production of carbon dioxide from C-1 of glucose (Table II). It appears, therefore, that glucose

Table II

GLUCOSE METABOLISM IN *Aerobacter aerogenes*

Growth medium ...	*Glucose*		*Glycerol*	
Additions ...	*None*	Mg^{2+}	*None*	Mg^{2+}
Glucose, μmoles	−1·8	−4·9	−1·5	−1·7
Gluconate, μmoles	+0·8	+3·5	—	—
Ketoglutarate, μmoles	+0·4	+0·6	+0·2	+0·2
Pyruvate, μmoles	+0·07	+0·4	+0·03	+0·02
CO_2, μmoles	+2·2	+2·8	—	—
O_2, μmoles	−2·7	−4·9	−3·1	−3·7
$^{14}CO_2$/[1-^{14}C]glucose	0·15	0·36	0·13	0·15

The experiments were carried out in Warburg vessels at 37° in phosphate buffer, pH 6·4, which contained Mg^{2+} at a concentration of 8×10^{-4} M where indicated. The last line of the Table presents the ratio of the radioactivity values of the isolated CO_2 and of the [1-^{14}C]glucose used as substrate.

induces the formation of an enzyme(s) which requires a high level of Mg^{2+}, and which permits the rapid degradation of glucose via gluconate, or presumably, phosphogluconic acid; at the same time glucose suppresses the formation of enzymes

required for the oxidation of pyruvate and ketoglutarate. The result of these effects is the accumulation of pyruvate and ketoglutarate, which are the precursors of most of the amino acids, and of gluconate or its phosphate, which is an important precursor of ribose and deoxyribose. Presumably, the high level of these essential metabolites is responsible for the rapid growth of the cells on glucose, and for the repression of the glucose-sensitive enzymes whose action would augment the already large pools of these metabolites.

It may, therefore, be concluded that enzyme repression is a general phenomenon and is not restricted to one particular class of enzymes.

The question now arises, by what mechanism the repressor, a small molecule, can prevent the synthesis of a specific protein. We have shown previously that glucose, or rather an intracellular product of its metabolism, exerts its effect on enzyme synthesis by interfering with the action of the enzyme-forming system, and not by preventing its formation, or by causing its destruction (Neidhardt and Magasanik, 1956*b*). These results would be in accordance with the hypothesis that enzyme biosynthesis is regulated by the interaction of the enzyme-forming system with repressors and inducers (Vogel, 1957*b*; Szilard, 1958, personal communication). According to this hypothesis [which in the case of β-galactosidase is supported by the brilliant experiments of Pardee, Jacob and Monod (1958)], the primary control of enzyme formation is exerted by the repressor, which may be thought to bind a newly formed enzyme molecule to the enzyme-forming machinery, and thus to prevent the further synthesis of enzyme molecules; the inducer would stimulate enzyme production by preventing the interaction of the repressor with the complex of enzyme and enzyme-forming system.

In the light of this hypothesis, repressors and inducers do not influence the formation of the enzyme-forming system, but control its action.

A large body of indirect evidence points to ribonucleic acid (RNA) as the enzyme-forming system (Brachet, 1955). It

has been clearly established that the cell contains more than one type of RNA, and that the "soluble" RNA, which in bacterial cells constitutes about one-tenth of the total RNA, plays an important part in the activation of amino acids for protein synthesis (Gros, 1958, personal communication). The bulk of the RNA exists in the form of ribonucleoprotein particles (Schachman, Pardee and Stanier, 1952), and it has been postulated that this material plays the rôle of a template in protein synthesis (Dounce, 1953): it orders the activated amino acids before they are polymerized to a protein molecule. The last step in protein synthesis is the removal of the protein molecule from the RNA template, and it is this process which repressors and inducers are presumed to affect. The rate of synthesis of an enzyme would depend on the ratio of the intracellular levels of its repressor and its inducer, which in turn would depend on the activity of this, as well as of other enzymes. Adjustments in enzyme levels would, therefore, not require the synthesis or destruction of the RNA template; the cell would at all times possess the machinery to form all the enzymes which it is genetically capable of producing. Even the drastic changes in the rates of synthesis of certain enzymes, which occur when the major carbon and energy source of the cell is changed, should not reflect the synthesis of new RNA templates, but rather the acceleration and retardation of protein synthesis by different existing enzyme-forming units.

We undertook to test this hypothesis by exploring the relationship of protein synthesis to RNA synthesis during the adjustment of *A. aerogenes* to changes in its environment.* In these experiments a mutant strain, 5-P-14, was used which required the amino acid, arginine, and the purine, guanine, for growth; it was therefore possible to limit the growth of the organism by restricting the supply of either of the two nutrilites, or of glucose, the major source of carbon and

* The experimental work described in this section was carried out by one of us (F.C.N.) at the Department of Bacteriology and was supported by U.S. Public Health Service Grant RG–3554.

energy. The effect of these restrictions on the formation of protein, RNA and deoxyribonucleic acid (DNA) during the

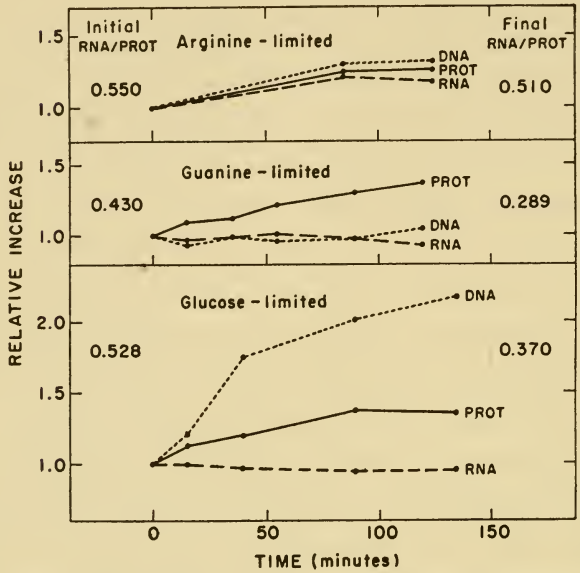

FIG. 1. The relative increases of DNA, RNA, and protein in cultures of *Aerobacter aerogenes*, strain 5-P-14, entering the maximum stationary phase because of limiting amounts of arginine, guanine, or glucose. Log phase cells were inoculated into the basal salt medium (Magasanik, Brooke and Karibian, 1953) containing the following compounds either in excess or limiting concentrations: glucose (0·2 per cent or 0·025 per cent), guanine (50 μg./ml. or 5 μg./ml.), and arginine (100 μg./ml. or 10 μg./ml.). The cultures were incubated aerobically at 37° and samples were taken at different times and analysed for RNA, DNA and protein by standard colorimetric procedures. The relative increases in these components are plotted against time, beginning when growth ceased to be exponential. The numbers to the left of the curves are the RNA: protein ratios at this time; the numbers to the right of the curves are the RNA: protein ratios of the final samples.

passage of the cell from the exponential to the stationary phase of growth is shown in Fig. 1. It can be seen that the lack of arginine affects equally the rates of formation of the

three cell constituents; guanine deficiency severely restricts the formation of the nucleic acids, but permits the protein to increase by about 30 per cent; exhaustion of glucose results in the cessation of RNA increase while protein and DNA increase by about 30 and 50 per cent, respectively. The net increase in protein after the exhaustion of the glucose from the medium, occurs at the expense of the accumulated products of glucose metabolism, which have been described earlier. This increase must represent in part the synthesis of new enzymes necessary for the further metabolism of these products, a synthesis which had been repressed during glucose degradation.

A similar situation occurs when the cells are grown in a medium that contains glucose as well as another compound capable of serving as a source of carbon and energy: as shown by Monod (1947), cells in such a medium grow first on the glucose, which suppresses the formation of enzymes required for the attack on the second carbon compound; a period of diminished growth, during which these enzymes are synthesized, separates the phase of growth on glucose from the phase of growth on the other compound.

The behaviour of the nucleic acid and protein fractions of the cell during the lag separating the two phases of this diauxic growth was investigated with the results shown in Fig. 2. During this period the cells made the transition from exponential growth on glucose (with a mean generation time of 45 minutes) to exponential growth on *myo*-inositol (with a mean generation time of 100 minutes). It can be seen that DNA synthesis continues at its original rate during the first part of the lag period, and then decreases to the new rate characteristic of growth on *myo*-inositol. Protein synthesis proceeds throughout the lag period, though at a slightly slower rate than DNA synthesis. On the other hand, no net increase in RNA could be detected throughout the period of the diauxic lag; RNA began to increase again only after the transition from glucose metabolism to *myo*-inositol metabolism had been accomplished. The failure to observe any net increase in RNA during the diauxic lag could conceivably be

the result of increased RNA breakdown masking a continued synthesis. For this reason the experiment was repeated, and a

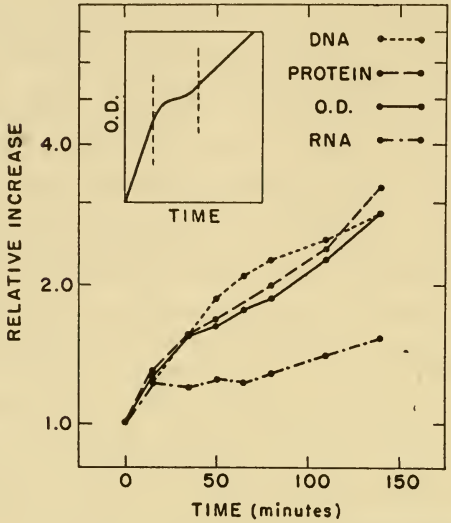

FIG. 2. The relative increases of DNA, RNA, protein and optical density in a culture of *Aerobacter aerogenes*, strain 5-P-14, during a diauxic lag. The cells were grown aerobically at 37° in the basal medium supplemented with guanine (50 μg./ml.) and arginine (100 μg./ml.), and containing glucose (0·06 per cent) and *myo*-inositol (0·2 per cent) as major sources of carbon and of energy. The inset to the figures describes the diauxic growth curve of this culture as followed by its optical density at 420 mμ. During the period included by the dashed lines in the inset, when the culture was adjusting from growth on glucose to growth on *myo*-inositol, samples were taken and analysed by standard colorimetric procedures for RNA, DNA and protein. The results are plotted in the main part of the figure as relative increases in these components against time. The increases are relative to the values at zero time, shortly prior to the beginning of the diauxic lag.

small quantity of [8-^{14}C]guanine was added to the guanine-containing culture at the beginning of the lag phase; the cells

were harvested at the end of the lag phase and their nucleic acids were fractionated. The molar radioactivity of the RNA-guanine and of the DNA-guanine was determined and compared with the molar radioactivity of the guanine present in the growth medium (Table III). It is evident that proportionally much less guanine was incorporated into RNA than

Table III

[8-^{14}C]Guanine incorporation into the nucleic acids of *Aerobacter aerogenes* during a diauxic lag

	RNA-Guanine	*DNA-Guanine*
Radioactivity of the isolated guanine relative to that in the medium (97,000 cpm/μM)	0·103	0·330
Increase calculated from the radioactivity	11%	49%
Increase calculated from colorimetric measurements	7%	40%

Cells of strain 5-P-14 were grown in a medium containing glucose and *myo*-inositol as major carbon sources with arginine and guanine present as essential growth factors exactly as described for Fig.2. [8-^{14}C]Guanine was added at the beginning of the lag phase and the entire culture harvested one hour later. Extraction of the acid-soluble pool and of the lipid fraction of the cells was followed by alkaline hydrolysis of the RNA, precipitation of the DNA and protein with acid, and then acidic hydrolysis of the DNA. The RNA-guanine was isolated as guanylic acid by electrophoresis, and the DNA-guanine was isolated as guanine by paper chromatography.

into DNA; the failure of the RNA to increase proportionally with DNA and protein during the diauxic lag thus appears to be due to an impairment of RNA synthesis, and not to an enhancement of RNA degradation. This result indicates that the synthesis of new types of protein, which is essential for the transition of the cell from one chemical environment to another, can proceed without the synthesis of an equivalent amount of RNA. Such a finding is in harmony with the concept that the synthesis of enzymes is regulated by the control of the action, and not of the formation of the protein-forming machinery.

The regulatory mechanism we have discussed so far serves to apportion the building blocks available for protein synthesis

(presumably activated amino acids) among the different units of enzyme-forming machinery (presumably ribonucleoprotein particles). The overall rate of protein synthesis, and therefore of growth, must depend on the intracellular concentration of the activated amino acids and on the RNA content of the cell.

It has been known for many years that the RNA content of a cell is not constant, but varies with the growth rate of the cell, although different authors have described this relation in quantitatively different terms (Brachet, 1955). We investigated therefore the relationship between RNA content and growth rate in *A. aerogenes*, the organism used in our studies of the regulation of enzyme formation. The results of a series of experiments in which we determined the RNA content (expressed as the ratio of RNA to protein) of cells growing at widely different rates are presented in Fig. 3. It can be seen that in rapidly growing cells ($k = 0 \cdot 6$ to $k = 1 \cdot 1$), the rate of growth, and therefore the rate of protein synthesis, is proportional to the total RNA content of the cell; however, in cells growing more slowly ($k = 0 \cdot 2$ to $k = 0 \cdot 6$), there is little variation of the RNA content with a change of growth rate.

It would, therefore, appear that when the rate of growth is severely restricted by the composition of the growth medium, it is the low concentration of activated amino acids, and not the insufficiency of the machinery, which limits the rate of protein synthesis: the cell is capable of increasing the rate of protein synthesis threefold (from $k = 0 \cdot 2$ to $k = 0 \cdot 6$) without a substantial increase in its RNA content. On the other hand, an increase in the rate of protein synthesis from $k = 0 \cdot 6$ to $k = 1 \cdot 1$, seems to involve an expansion of the protein-forming systems of the cell. The proportionality of the rate of protein synthesis and of the total RNA content of the cell in this range would seem to imply that the concentration of the activated amino acids is sufficiently high to saturate the protein-forming machinery; the rate of protein synthesis is, therefore, a function only of the *amount* of protein-forming machinery which the cell contains.

It is physiologically advantageous for a cell to be able to

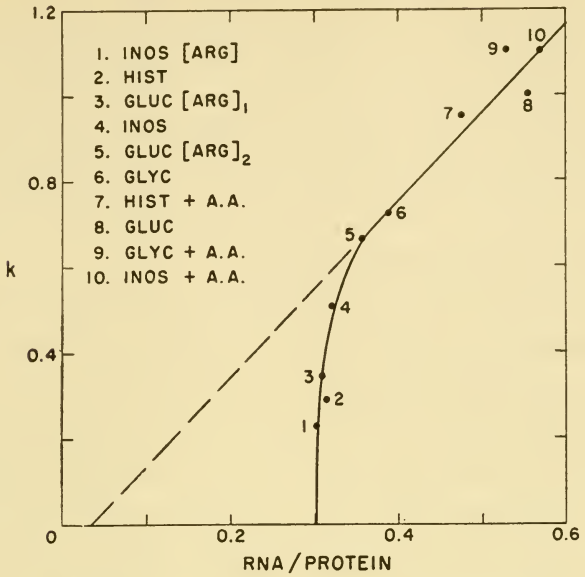

FIG. 3. The relationship between growth rate and RNA content of *Aerobacter aerogenes*, strain 5-P-14. The growth rate constant, k (hours^{-1}), is defined elsewhere (Neidhardt and Magasanik, 1956*b*), and the RNA content is expressed as the RNA: protein ratio based on colorimetric measurements. Each of the numbered points in the figure represents the average of at least two experiments, analysed in duplicate. The cells were grown (aerobically, at 37°) in the various media for at least three generations before any samples were taken. Except where indicated otherwise, all of the media contained basal salts supplemented with excess amounts of guanine (50 μg./ml.) and arginine (100 μg./ml.). The major carbon and energy sources, supplied at 0·2 per cent final concentration were: (1) *myo*-inositol, with arginine supply limited by the chemostat; (2) L-histidine; (3) glucose, with arginine supply limited by the chemostat; (4) *myo*-inositol; (5) glucose, with the supply of arginine by the chemostat greater than in (3) but still limiting; (6) glycerol; (7) L-histidine plus hydrolysed casein; (8) glucose; (9) glycerol plus hydrolysed casein; (10) *myo*-inositol plus hydrolysed casein.

vary its content of protein-forming machinery. Bacteria handle their resources with great economy: we have seen that

enzymes are no longer made when they have become superfluous or redundant. The same economy seems to apply in part to the production of RNA. The cell growing on *myo*-inositol (Fig. 3, point 4), which is not metabolized rapidly enough to give maximal growth, uses a smaller proportion of its resources to make RNA than a cell growing on glucose (Fig. 3, point 8); a larger portion of the energy and the building blocks produced by the degradation of *myo*-inositol, is, therefore, available for protein synthesis.

The observation that the composition of the growth medium affects the RNA content of the cell suggests that the rate of RNA synthesis is sensitive to the concentration of some intermediary product(s) of metabolism. Several lines of evidence suggest that the rate of RNA synthesis is controlled by the concentration of the amino acids in the cell: (1) cells deprived of an amino acid required for protein synthesis immediately cease to synthesize RNA. This well known fact is illustrated in Fig. 1, where it can be seen that arginine deprivation prevents the synthesis of RNA as well as of protein. (2) The synthesis of RNA without concomitant synthesis of protein, which occurs in chloramphenicol-inhibited bacteria, depends on the presence of all of the amino acids required for protein synthesis (Pardee and Prestidge, 1956; Gros and Gros, 1956; Yčas and Brawerman, 1957). (3) The addition of a mixture of amino acids to cultures of *A. aerogenes* growing at a slow rate on *myo*-inositol or glycerol (Fig. 3, points 4 and 6), increases their rate of growth and their RNA content to a level which seems maximal for this organism (Fig. 3, points 9 and 10).

Let us therefore assume that amino acids, or perhaps their activated derivatives, are essential not only for the synthesis of protein, but also for the synthesis of RNA and, furthermore, that the protein-forming system has greater affinity for these metabolites than the RNA-forming system; a ready explanation for the observed relationship between the growth rate and the RNA content of the cell is then at hand. In a rapidly growing cell ($k > 0{\cdot}6$, see Fig. 3), the level of activated amino acids is sufficiently high to saturate the protein-forming

system, but not high enough to saturate the RNA-forming system. Consequently, any change in the composition of the medium which increases the level of the activated amino acids in these rapidly growing cells will cause an immediate increase in the rate of RNA synthesis; the rate of protein synthesis will not be directly affected by the higher level of the activated amino acids, but will increase gradually with the rising RNA content of the cell.

The assumption that the RNA-forming system has less affinity for amino acids than the protein-forming system would also explain the observation, illustrated in Figs. 1 and 2, that after the exhaustion of glucose, protein synthesis continues at a diminished rate, while RNA synthesis ceases. It has been shown by Mandelstam (1958) that in *Escherichia coli* the intracellular concentration of amino acids decreases markedly during the transition of the cell from the exponential phase of growth on glucose to the stationary phase; a point will be reached at which the level of the amino acids will be sufficient for protein synthesis, but too low to permit the synthesis of RNA to proceed at a measurable rate.

We may summarize the concepts of the regulation of cell growth and composition discussed in this essay in four statements.

(1) The genome, i.e. the DNA fraction of the cell, possesses all the qualitative information required for the synthesis of every protein that the cell is capable of making.

(2) This information is transmitted to the actual protein-forming machinery, which consists of ribonucleoprotein particles; these act as templates for the construction of the protein molecules. The cell contains at all times an equal number of templates for every species of protein that it is genetically capable of making. However, the rate of synthesis of all templates depends on the intracellular concentration of the amino acids required for protein synthesis (or of their activated derivatives). Consequently, a cell growing in a rich medium will have a greater capacity for the synthesis of all proteins, than a cell growing in a poor medium.

(3) The overall rate of protein synthesis depends on the amount of RNA the cell contains, and on the intracellular concentration of the activated amino acids.

(4) The rate at which an individual protein is formed depends on the activity of the corresponding units of protein-forming machinery; this activity is controlled by the interaction of the RNA template with specific repressors and inducers, which are in general products and substrates, respectively, of the enzyme-protein whose production they regulate. This automatic adjustment provides the cell with the enzymic composition best suited for growth in a particular environment.

It is hardly necessary to stress that most of the evidence on which these concepts are based is preliminary and indirect, and will require confirmation by decisive experiments with isolated cell fractions. However, it would seem to us that the formulation of a unified hypothesis for the regulation of cellular growth and composition at this time may be useful in planning future experiments. The clear recognition of the feebleness of the foundations on which the hypothesis rests should enable us to pursue this work with an open mind.

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DISCUSSION

McIlwain: Dr. Magasanik, you explained that the phenomenon of enzyme repression could have some advantage for the cell. I wonder whether you have tackled this experimentally in the sense of putting into competition cells which for instance have, and others which do not have, the mechanism for repressing galactosidase by glucose, etc. This is related to the earlier discussion concerning enzyme activity; and matters such as the K_m of the enzyme, the mutual adjustment between K_m of the enzyme and the level of substrates available to the organism or to the enzyme in the cell, are involved.

Magasanik: It would be very nice to have such mutants but I don't know of any cell which can be induced and is not subject to repression. We believe that even in a cell that is not strongly subject to repression by glucose, there is still some repressive mechanism active, but it is set at a higher level; we would have to obtain mutants that have a very ineffective mechanism as against those with a very good repressive mechanism.

Pardee: Dr. Novick (personal communication) told me of a mutant which may serve this purpose. Bacteria can be inducible or constitutive: inducible bacteria need an inducer to make the enzyme, whereas a constitutive mutant makes the same enzyme at a very high rate all the time without needing an inducer. These bacteria apparently lack the repressor, therefore they do not need an inducer. Novick has compared the growth rate of one of these constitutive organisms (which is making about 10 per cent of its dry weight in β-galactosidase) with that of the wild type which is not making this enzyme. The experiment is done in the absence of galactose when there is no function for this enzyme. One organism makes the enzyme, the other does not, and the one that does not make the enzyme grows about 20 per cent faster than the one that does make the enzyme in this case. It is a single-step mutant; there is probably only one genetical change in the cell. Therefore, there is some possibility that the slower growth-rate is due to the forced formation of a lot of extra protein in this organism.

McIlwain: I should like to see the more ecological experiment of the two being put together.

Pardee: This has been done in what is called a chemostat, something

like what Prof. Lynen has described, where medium is continually dripped in and bacteria grow under steady conditions for many days. Many experiments have been done to show that the organism that grows faster wins out in a short time.

Siekevitz: In mammalian organs, as has been found by many people, the turnover of the RNA is not necessary for protein synthesis. Also, the RNA in the nucleoprotein particles that I mentioned earlier turns over very slowly, if at all, whereas their determinable protein does so very rapidly.

Coxon: Is it justifiable to deduce from what Dr. Magasanik said, that the number of potential inducers is limited by the pre-existing enzyme-forming systems in the cell? Presumably there is only a limited class of compounds that could act as inducers if there has to be a pre-existing enzyme-forming system which, in turn, is repressed in the process of induction.

Magasanik: Yes. It is now thought that the inducer acts on a pre-existing enzyme-forming system. This view is relatively new. Earlier, it was thought that the inducer itself became part of the enzyme-forming system. However, it is known that the formation of each inducible enzyme is genetically controlled; unless the gene is present, the inducer is incapable of effecting the formation of the enzyme. The gene is thought to have the information which determines the amino acid sequence of the enzyme protein, and to transmit this information to the enzyme-forming system. What additional information, essential for the construction of the enzyme-forming system, could the inducer provide? I think the answer is: none. It is therefore tempting to speculate that the enzyme-forming system is formed independently of the presence or absence of the inducer, but that the inducer acts on the enzyme-forming system, presumably by competing with a specific repressor. According to this hypothesis, the cell will contain at all times all the enzyme-forming systems it is capable of producing, but the rate at which each of these systems turns out the appropriate enzyme will depend on the intracellular levels of the corresponding inducers and repressors, and these, in turn, will depend on the composition of the growth medium.

Lehninger: Dr. Magasanik, Monod found that he could induce an enzyme with a substance which does not necessarily act as a substrate. Can you repress enzyme formation with something that is not technically a product of the enzyme that you are repressing?

Magasanik: Yes. Mr. A. P. Levin in our laboratory had found (unpublished data) that the guanine analogue, azaguanine, represses the formation of inosinicase and of inosinic acid dehydrogenase in the same way as guanine. This effect of azaguanine is distinct from any general inhibitory effect of this compound on protein synthesis, since the formation of other enzymes, e.g. glycerol dehydrogenase, is not inhibited under these conditions. I should add that analogues of amino acids may mimic the inhibitory action of the corresponding amino acid on an early step of their biosynthetic pathway. This would explain why an organism cannot overcome the growth-inhibitory effect of such an analogue by producing

more of the corresponding amino acid by synthesis *de novo*. Dr. H. S. Moyed has found evidence for this view (personal communication) in experiments with analogues of histidine and of tryptophan. In both cases the analogue inhibits the same early enzymic step as the corresponding amino acid. In a mutant which has been selected for resistance to the histidine analogue, this early reaction, the condensation of AMP (or ATP) with activated ribose phosphate, is no longer inhibited by either histidine or by the analogue. It would appear, therefore, that ability to interfere with the synthesis *de novo* of the corresponding amino acid is the attribute which makes an amino acid analogue an effective growth inhibitor.

Pardee: That has been shown in several cases, where the analogue prevents the formation of early intermediates in the biosynthesis of the normal compound of which it is an analogue.

Lehninger: Cytidine triphosphate inhibits the formation of ureidosuccinic acid; can cytidine monophosphate be a repressor of the enzyme which makes ureidosuccinic acid?

Pardee: That is quite possible. We cannot tell whether the repressor is cytidine triphosphate, cytidine monophosphate or uridine monophosphate, etc. We added uracil and it made something which serves to repress the formation of this enzyme. Incidentally, the uracil analogue, azauracil, acts in the same way but less efficiently.

Racker: The idea of the displacement of a repressor by an inducer appeals to me; they could both compete for a common site of the template.

Magasanik: There is no decisive evidence that the inducer is not part of the template. The evidence is philosophical. If the gene already has the information, why is the inducer needed?

Pardee: Monod's argument is that, if an inducer is added, the bacteria start forming enzyme with no appreciable lag, i.e. within a minute. It is hard to see how bacteria can suddenly make a lot of new specific templates within one minute; one would expect an autocatalytic phase.

Magasanik: I realize that it would be difficult to discuss here at this time the brilliant experiment of Pardee, Jacob and Monod on the formation of β-galactosidase in zygotes. The results of this experiment provide strong evidence that the primary event in the control of the formation of β-galactosidase is repression, i.e. inhibition of a pre-existing enzyme-forming system.

Pardee: We have been working recently on bacterial recombination (Pardee, Jacob and Monod, 1958, *loc. cit.*). The general principle, which has been demonstrated by others, is that there are two kinds of bacteria, male and female, in that the male bacterium can inject its genetical material but not its cytoplasm into the female bacterium. One can study a single gene, in our case the β-galactosidase gene, in such a system where the male bacterium has this gene in the active form and the female is inactive in ability to form the enzyme. Originally the gene is inside the male bacterium; when it is mixed with the female bacterium the gene is injected. Also, the female bacterium is constitutive: if it were able to make β-galactosidase it would make this enzyme all the

time without having an inducer present; but it cannot make β-galactosidase because it is altered in its galactosidase gene. The male is inducible.

What happens when the gene which has the ability to make β-galactosidase gets into the cytoplasm which is constitutive? Do you find enzyme formation or not? As soon as this gene enters the cytoplasm it starts to make enzyme at full speed, within one or two minutes. This result argues against any very elaborate synthesis of a lot of templates. Consider the constitutive cytoplasm: the result means that the cytoplasm is ready to permit the formation of enzyme by this gene. There are two possible explanations. One is that the cytoplasm is loaded with inducers which are made inside the cell, so that as soon as the gene enters, the inducers start acting. The other explanation is that the cytoplasm is empty: there is a repressor in inducible bacteria which prevents enzyme synthesis, but in constitutive bacteria the repressor is missing so that when the gene escapes from the repressor it starts making the enzyme. It seems that the repressor idea is the right one, because when you inject the galactosidase-positive gene you also introduce the inducible gene at the same time, and in about 30 minutes after this gets in, conditions are changed so that the cell is no longer constitutive, but has become inducible. You have to add an inducer now to get the bacterium to continue making enzyme. Material has been injected, from male to female, which creates something new that limits enzyme formation. Both kinds of genes, constitutive and inducible, exist in one cell after mating, and the dominant gene is the inducible one. This inducible gene makes something, the repressor, whereas the constitutive cell has no need for any inducer and, therefore, presumably an inducer would not be part of a template.

GENERAL DISCUSSION

Dickens: Before opening this general discussion in summary of the symposium and on future research, I should like to take the opportunity to put on record, on behalf of us all, our sincere gratitude to Sir Hans Krebs for conceiving the whole idea of this symposium, and for ensuring, with the enthusiastic support of Dr. Wolstenholme and his staff at the Ciba Foundation, that it would be a great success, as I am sure we all feel it has been.

At this stage I might mention the very high density of Nobel laureates present which, even in the distinguished history of the Ciba Foundation, must be exceptional. I calculate it to be ten per cent of those present!

Among the prizes I should like to award, the first one would go to Prof. Lehninger for what I believe to be an historic contribution to the mechanism of oxidative phosphorylation. This is one of the great things that have come out of this meeting. Also one to Prof. Chance for solving 25 simultaneous equations and, what is more, getting sense out of the answer. Dr. Hess tells me that the right answer came up the very first time they did this, which must be a remarkable feat. I am quite sure that this step marks a taking off point on the journey into the future of biochemistry.

Another goes to Prof. Lynen and Dr. Johnson, who simultaneously in 1941 recognized with quite rare insight the vital importance of intracellular inorganic phosphate in control of metabolism; and to Dr. Siekevitz who showed us some beautiful electron micrograph pictures, which he was careful to credit to Dr. Palade. His own interpretation of these complex and fascinating diagrams gave us a great deal of matter for thought, especially the possibility that the enzymes and coenzymes can move about to different positions on the endoplasmic reticulum under hormonal and other controls; also the fascinating possibility that the nucleus, because of its communication with the exterior, may be outside the cell instead of inside! This is a thrilling possibility—that we are dealing with an extracellular particle when we are dealing with the nucleus.

In this connexion I would award a black mark to whoever is the inventor of one of the ugliest words in the English language, namely "compartmentalization." We should consider substituting "segregation" or some similar word, if this is not thought to have some less agreeable connotation. It must then be the keyword for this symposium, since so much emphasis has been rightly placed on the

cellular compartments for ATP, inorganic phosphate, DPN, TPN, enzymes, substrates, and so on.

Krebs: It has been suggested that we might discuss the use of the term "feedback". The word feedback comes originally from purely physical systems. The German term is "Rückkopplung". It originated in the 1930's in the field of electronics and is used for many physical devices. If we use it for chemical systems we extend the use of the term, but we all know what we mean. The essence of feedback is that the process, as it progresses, creates conditions which are unfavourable for the further progress of the process and cause it to stop. This stoppage in turn leads to environmental changes, because we are dealing with a system which is in equilibrium—and that is a fundamental issue—but a system in a steady state where various processes happen continuously. Therefore, the stoppage of any particular process would automatically lead to further changes which again favour the particular process and make it go on faster. One of the simplest feedback devices is a thermoregulator, fed by gas. A device is incorporated in the gas supply (an expanding fluid) which cuts off the gas supply by the effect of the gas, i.e. heat.

Magasanik: Is that what one would call, strictly speaking, negative feedback?

Krebs: Yes. Engineers speak of negative feedback when an increased output decreases the input, and of positive feedback when an increased output increases the input. Thus, the inhibition of pyrimidine synthesis by cytidylic acid is negative feedback. On the other hand, the stimulation of respiration by ADP and/or phosphate may be looked upon as positive feedback: in this case the greater the rate of ATP utilization, i.e. the rate of formation of ADP and phosphate, the greater becomes the rate of respiration and oxidative phosphorylation. I feel that it is generally unnecessary, in the chemical systems which we are considering, to mention the "sign" of the feedback mechanism. (For further reference, see Tustin, A. (1953). The Mechanism of Economic Systems. London: Heinemann; Flanagan, D., and Svirsky, L. *Eds.* (1957). Automatic Control, *Sci. Amer.* London: Bell.)

Chance: A simple diagram (Fig. 1A) should help to explain what negative feedback may signify to workers in the field of electronic circuitry. The input to the electrical system, designated E_{in}, causes a flow of electrons along the input conductor. These electrons then flow into another conductor at a potential—E_{out}. The amplifier provides a potential E_{out} of such magnitude that the electron flow through R_2 is exactly equal to the electron flow through R_1. If these electron flows should be unequal, the amplifier would adjust

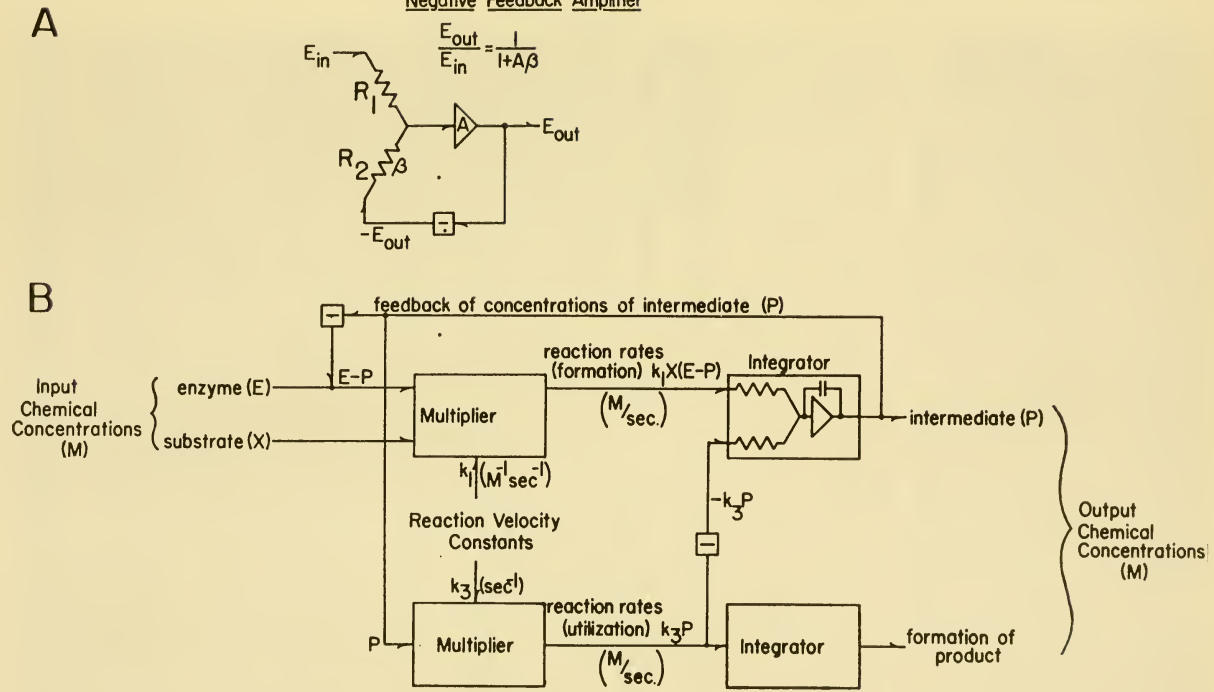


FIG. 1A and B (Chance.) (Expt. no. MD-72)

E_{out} so that the inequality would be abolished. Under these conditions there is a precise relationship between the output and input potentials, as given in the figure. The potentials are equal when $A\beta$ is large.

The chemical analogue of the electrical feedback amplifier is difficult to draw, as illustrated by two examples. If the voltages represent the analogues of chemical concentration, the system requires a chemosynthetic system as an amplifier (A) and a device for expending the input chemical as a feedback network (β). Another approach is to apply the formal relationship for the feedback amplifier to the Michaelis-Menten equation by comparing

$$\frac{E_{out}}{E_{in}} = \frac{1}{1 + A\beta}$$

with

$$\frac{p}{e} = \frac{1}{1 + K_M/x}$$

The negative feedback system represents the Michaelis-Menten system if $A\beta = K_M/x$ where, for example, $A = 1/x$ and $\beta = K_M$. This appears to be an unrealistic model, for amplifier gain is equated to reciprocal concentration.

The fruitful analogue of feedback in a metabolic control system is afforded by the analogue of the mass-law equations for the system (Fig. 1B). The inputs to this analogue are chemical concentrations, E, X which are multiplied to give products of concentration (E–P)X where bimolecular reactions are involved. Rates of reaction are obtained from the products of the concentrations and reaction velocity constants, and thereby rates of formation and rates of utilization of intermediates can be specified ($k_1X(E–P)$, k_3P). Integration of the differences between rates of formation and rate of utilization will give the chemical concentrations of the intermediate (P) which may be increasing, decreasing, or steady (as in the steady state); this can be subtracted by a feedback connexion to regulate the amount of free enzyme available. Thus, chemical feedback in a mass-law system is obtained from the integration of differences between rates of formation and rates of utilization.

Such a system bears little formal relationship to the block diagram of the negative amplifier (Fig. 1A). In fact, only a remote resemblance is indicated by the integrator circuit itself which is a type of feedback amplifier in which the feedback current is supplied through a condenser.

In summary, feedback in a chemical system is not directly comparable to the negative feedback of electronic circuitry; none of the

obvious features of gain stabilization (Fig. 1A) or more favourable dynamic response, etc., apply, and it is probable that a quite different set of theorems on feedback in chemical systems will be evolved. Here and now, I wish to establish that the chemist and the engineer refer to very different types of mechanisms.

In a chemical reaction sequence, it is important to identify the site of metabolic control in terms of the specific control chemical. We can illustrate this by a system in which ADP is controlling.

$$\text{ADP control:} \quad \text{ADP} \xrightarrow{\text{fast}} \text{ATP} \xrightarrow{\text{slow}} \text{ADP} \qquad (\text{Fig. } 1C_1)$$

ADP is utilized in a rapid reaction, forming ATP through, e.g., the respiratory chain. ATP is utilized in a slow reaction, which is rate-controlling. The control substance, in so far as ADP utilization is concerned, is ADP, and its site of action is the fast reaction. If the slow step is saturated with respect to ATP, changes of the ATP concentration have no effect on the flow of metabolism. However, any other system contributing to the rate of formation of ADP will participate in the control of respiratory metabolism by alteration of the ADP level.

With a simple change of rate constants, the same system of reactions can demonstrate ATP control.

$$\text{ATP control:} \quad \text{ATP} \xrightarrow{\text{fast}} \text{ADP} \xrightarrow{\text{slow}} \text{ATP} \qquad (\text{Fig. } 1C_2)$$

If ATP is expended in a fast reaction and ADP in a slow reaction, the regeneration of ATP is slow. Thus, the balance of formation and utilization reactions determines which substance is rate-controlling.

In these examples, metabolic control is exerted exclusively by the slow step of both control mechanisms. In the cell the control chemical may be produced and utilized by a variety of reactions. The sum of the rate of these reactions is conveniently represented (Fig. 1B) by the steady state concentration of the control chemical (ADP in Fig. $1C_1$). For this reason it is much simpler to focus our attention on the steady-state concentration of this control substance, since it represents the net result of a large number of processes that cannot be measured individually.

A single enzyme reaction affords an example of the regulatory metabolic system. This is an especially useful example since many properties of a single enzyme system are familiar to us. In this case, the enzyme is utilized to form the enzyme-substrate intermediate. If this is a fast reaction and if the intermediate is converted back to the enzyme in a slow reaction, the flow of substrate

through the system is regulated by the enzyme concentration, provided an excess of substrate is present.

$$E + S \xrightarrow{k_1} ES$$

$$ES \xrightarrow{k_3} E + P$$

(Fig. 1D)

Enzyme control: $E \xrightarrow{\text{fast}} ES \xrightarrow{\text{slow}} E$

Intermediate control: $ES \xrightarrow{\text{fast}} E \xrightarrow{\text{slow}} ES$

This is a basic example of a metabolic system regulated by enzyme concentrations and it may therefore cover some of the cases referred to by Dr. Magasanik. It is obvious that synthesis of the enzyme will increase the metabolic flux.

The last line of Fig. 1D represents an enzyme system operating at low substrate concentrations. Here the enzyme-substrate compound is the rate-limiting component and its breakdown is the rate-limiting reaction.

It may be mentioned in passing that substrate control of an enzyme system does not directly involve a feedback, since the product of the enzyme activity is not identical with the substrate. However, if subsequent reactions produce substrate, the type of control considered here may be obtained.

The differential equation for a single-enzyme system also sheds light upon the general properties of metabolic control:

$$\frac{dp}{dt} = \underset{\text{formation}}{k_1 (e-p)x} - \underset{\text{utilization}}{k_3 p}$$

(Fig. 1E)

$$\frac{dx}{dt} = \underset{\text{utilization}}{k_1 x(e-p)}$$

The steady state concentration of the rate-limiting intermediate is dependent upon reaction rate terms involving the products of various concentrations. For a single-enzyme system, only the product of the enzyme and the substrate concentrations are involved and the rate of utilization of the intermediate is proportional to its concentration; for more complex systems, other terms are involved. The overall balance between rate of formation and rate of utilization

determines whether a particular reaction sequence is controlling and whether it is the concentration of the free enzyme or the concentration of the enzyme intermediate that is the control substance.

Potter: Could this be extended directly to the situation of the enzyme-forming system in which—in a sense—one does have an amplifier, if the amount of enzyme is increased by net synthesis, not just the amount of free enzyme? I think this is quite analogous.

Chance: It is clear that under conditions where the enzyme concentration, E, is the control substance, an enzyme-forming system would accelerate the metabolism. However, I do not feel that the analogy to the electrical amplifier is an especially useful one.

In Fig. 1B, for instance, enzyme synthesis would increase the concentration of reactant E, and hence would speed the rate of formation of the intermediate over the metabolism. On the other hand, if an enzyme were degraded, it would simply subtract from the input to the control system and that would depress the amount of enzyme.

Potter: It would be worth while to discuss the situation in which many enzyme systems, which have their own substrate, have a reactant in common, e.g. ADP. When one of these systems uses up a common substrate which affects the rate of reaction of other systems, can this properly be referred to as feedback and, if not, what should we call it?

To get down to cases, our Chairman in his opening discussion pointed out that if one member of the citric acid cycle is present in large amount, it will repress the oxidation of the other members of the cycle. It may do this by means of a number of them using DPN, for instance, so that the one which is present in large amount takes over the DPN and makes it less available for the others. Can the Michaelis constant be incorporated, and what should it be called? It is really competition.

Chance: It is appropriate to refer to the ADP control system mechanism of Fig. 1C in which ADP is utilized in a rapid reaction and is regenerated in a slow reaction. It is possible that several independent enzyme systems contribute ADP in parallel; the total rate of production of ADP would be the sum of the individual rates. Indeed, as in the case of glycolysis and respiration, there may be more than one system expending the ADP supply. It is, therefore, true that as one of the systems uses up the ADP, the other systems will also be affected. To the extent that any metabolic regulation of the type discussed here can be called a feedback, it is also appropriate to designate a multi-enzyme system in the same way (Potter, V. R. (1957). *Univ. Mich. med. Bull.*, **23**, 401). Perhaps it is possible to represent the DPN-linked dehydrogenases of the citric acid

cycle as a group of DPN-utilizing enzymes involving this substance in a rapid reaction and that the respiratory chain regenerates DPN in a slow reaction:

$$\text{DPN} \xrightarrow{\text{fast}} \text{DPNH} \xrightarrow{\text{slow}} \text{DPN} \qquad \text{(Fig. 1F)}$$

The dehydrogenase having the largest velocity constant for the utilization of DPN would be able to operate at the lowest DPN concentration and would therefore win in competition with the other members of the cycle. When one member of the cycle has won in the competition and becomes the main source for DPN reduction, it is then easy to apply the concept to the Michaelis affinity for a single enzyme system. But in the competitive reaction, the important quantity is the rate term k_1 [DPN] [E].

Incidentally, I neglected to mention previously that when the rates in the utilization and regeneration of the intermediate are equal, there can be no control by that particular substance, as indicated in Fig. $1C_3$:

$$\text{No control:} \qquad \text{ADP} \xrightarrow{\text{equal}} \text{A} + \text{P} \xrightarrow{\text{equal}} \text{ADP} \qquad \text{(Fig. } 1C_3\text{)}$$

Racker: I believe one could set up an experiment in glycolysis which may resemble a positive feedback mechanism. For each pyrophosphate bond of ATP expended there is a net return of 2 high-energy bonds formed during glycolysis. In the presence of low ATP and high AMP the rate of glucose utilization should therefore increase until maximum efficiency is reached at saturating ATP concentrations.

Chance: Not really, because in electrical systems positive feedback immediately leads to instability (unless it is counteracted by another negative feedback). (See Fig. 1A.)

Racker: But there is net synthesis of 2 pyrophosphate linkages for each glucose which is fermented.

Chance: I still have difficulty in equating chemical synthesis to electronic amplification. I suppose this is indicated by Fig. 1A, where the output of the amplifier is fed back to the input as a negative concentration. Chemicals do not simply disappear; they are always converted to something else.

Lehninger: To quote a good example: suppose you start with pyruvic acid and the overall processes of its disappearance; you need coenzyme A to make this. Let us say that some oxidation product, past coenzyme A, is utilized as a building block for making

coenzyme A, for making the catalyst. You are removing part of the flux of the carbon through the scheme to go back and make catalyst and to make your overall rate go faster. Can you call that a positive feedback? Supposing the enzyme was rate-limiting.

Chance: This question is similar to Prof. Potter's concerning enzyme synthesis. It is certainly true that a synthesis of the rate-limiting intermediate will cause an increase of the rate of overall metabolism. In addition, an increase in the concentration of the substances involved in such a synthesis will further increase the rate. I will agree that this is positive feedback of the type that may exist in the chemical system but, as I have stated above, the positive feedback in the electrical system leads to instant instability. Alternatively, the effect could be regarded as an amplification.

Racker: ATP formation in glycolysis occurs at later stages, far removed from the initial steps of utilization. Why is that not a positive feedback?

Chance: Again I wonder whether it is really the analogue of the electrical network in which it is possible to have positive and negative voltages; in the direct chemical analogue you would need positive and negative ATP, but we do not have a negative ATP in the chemical system.

Racker: Can you consider ADP to represent "negative ATP"?

Chance: I do not think this would be helpful. The chemical analogue of the electrical feedback system avoids this difficulty and satisfactorily represents the mass-law equations.

Siekevitz: Negative and positive feedback may be all part of the same picture if you consider the segregation of molecules. Product inhibition produces negative feedback if all the reactants are in one compartment, but a positive feedback may occur when the product is segregated across some barrier and cannot get back. Thus, the reaction goes on to completion. The controlling factor may, thus, have to do with membrane permeability.

Chance: For a simple mass law system, the concentration controls the rates.

Siekevitz: It controls the rates if it is in the same compartment, but what if the product always comes out into another compartment?

Chance: If segregation of the product by compartmentation occurs, then this substance will become a rate-limiting intermediate, since no regeneration occurs. This is, therefore, probably not a feedback control of the type we are discussing here, unless there is a leak from the compartment. I am sure I am being too rigid about all this.

Krebs: That is all to the good.

Lipmann: I am at a loss if this is put into equations. But I feel that most of what we call feedback mechanisms may be rather simply, and without further formalization, described as the regulation of supply and demand. The regulation of the rate of respiration by the acceptor effect, the availability of ADP, is a good example of that.

Krebs: The term feedback, as used by engineers, has been defined as control of input by output.

Potter: I should like to hear Dr. Pardee and Dr. Magasanik comment on some of these points. They both have excellent examples of chemical feedback.

Pardee: I am at present more interested in experimental results and prefer not to attempt a definition.

Magasanik: There seems to be one difference between the energy-yielding systems which we have discussed during the last two days, and the systems involved in biosynthesis of amino acids, purines, etc. which we have discussed today; and that is that the control in the former case is through equilibrium reactions, reactions that can essentially go forward and backwards, and that it is the level of the substrates which controls the rates of the reactions; whereas in the biosynthetic sequences many steps are irreversible: the reactions of biosynthesis are being driven towards the formation of protein. There, of course, since a regulation by equilibrium is not possible, the regulation is effected by having the products act as specific inhibitors of earlier steps. This seems to be a fundamental difference which may have physiological significance for the cell.

Krebs: I do not agree that there is a fundamental difference. All these control mechanisms have an aim, namely to maintain a steady state system. In the energy-yielding reactions, the aim is to keep a supply of ATP. In the biosynthetic reactions, it is to supply a basic pool of e.g. amino acids. The supply comes to a stop when the pool of amino acids is sufficient to maintain the synthesis of protein.

Lipmann: I think that a higher mode of construction has to go into the control mechanism. When the end product of a reaction series inhibits an enzyme which is quite far back along the line, this has nothing to do with equilibrium; it is just construction.

Krebs: What you say appeals to me. If we assume that every catalyst constitutes a feedback system (as Prof. Chance has suggested), then we rather dilute the concept of feedback.

Chance: The point is that the top equations are probably similar to the bottom ones. The relationship is that the controlling intermediate is behaving mathematically like an enzyme, i.e. it is utilized and is regenerated in a cyclic fashion, it is not lost in the

process, and it is in minimal concentration at one point in the cycle. That cycle can be as long as you wish. It can extend from oxygen to glucose to oxygen.

Lipmann: On the other hand, in this long-chain reaction where there are irreversible steps in between, the cycle is not really reversible. The E is actually E plus some inhibitor. It is no longer in equilibrium in any thermodynamic sense. Something has been formed far back which now goes back and hooks on to the enzyme. I wonder if there is any cyclic reaction.

Potter: Up to this point we have been discussing straight-line operations in which something goes along an unbranched pathway. The essence of the problem is the fact that there are alternatives for these metabolites. A given metabolite can go in two or more different directions, and anything that is done to one reaction results in an effect on the other reactions. We can stimulate one by inhibiting the other one. This is our basic problem in metabolic control: how do we effect this competition? When we consider the bacterial synthesis of pyrimidines from carbamyl aspartic acid, which is converted through many intermediates into uridylic acid and to cytidylic acid, as has been shown by Dr. Pardee, and consider how this is controlled in the bacterial cell we may say that uracil can produce uridylic acid, which can form cytidylic acid, and the latter by negative feedback can shut off the synthesis of itself from aspartic acid. The important point is that in either case the cell gets cytidylic acid and grows, so that this particular feedback mechanism is simply a device for growing under economical conditions, choosing whether growth shall be from uracil *or* from carbamyl aspartic acid.

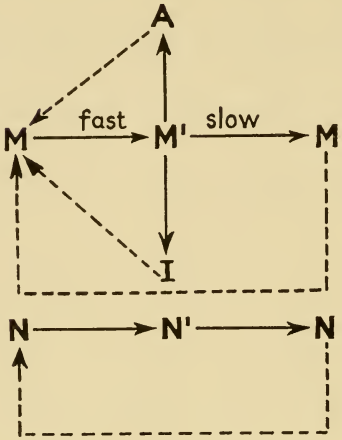
Another important point is that when a liver cell is confronted with both aspartic acid and uracil it still does not make more liver cells; it somehow decides not to make thymidylic acid even though it has both uracil and aspartic acid. One of the ways in which it can do this is by disposing of some intermediate in such a way that thymidylic acid synthesis does not take place.

My first point was that the essence of metabolic control is the choosing between alternatives. Finally, in animal tissues we have to prevent certain things such as DNA from being synthesized in certain tissues, even when all the normal distal building blocks are present.

Krebs: The choosing of alternatives could be interpreted as feedback.

Chance: If a metabolic system is to be able to select between alternatives and at the same time to be maintained "under control" in the sense in which we have been discussing it here, there must be suitable control mechanisms for the two pathways which we will

here identify as involving the control metabolites M and N, respectively:



(Fig. 1G)

If metabolism along pathway M involves, in addition to the usual control mechanisms, the formation of inhibitors (I) or activators (A) in branched pathways, it is easy to see that the formation of such substances, either from M′ or from any other source in the cell, could readily change the balance of metabolism through the M and N pathways. An ideal substance for shifting metabolism from one pathway to another would be one that inhibits pathway M and activates pathway N. It is my purpose, however, to emphasize that any discussion of metabolic control presumes that there exists in the cell a substance whose concentration controls the metabolic pathway of interest. From that point onwards, pathways capable of activating or inhibiting the control system can easily be visualized.

Magasanik: We have studied one case where this sort of branching, and its control, can be nicely seen. In certain species of bacteria, for instance *Aerobacter aerogenes*, and in certain animal tissues, e.g. liver, histidine can serve two functions: it can be incorporated directly into protein, or it can be degraded to glutamic acid, which can be used as a source of energy and of non-specific building blocks. We can see that the cell is therefore faced with the problem of whether to use the histidine for protein synthesis, or to break it down. We found several years ago (Magasanik, B. (1955). *J. biol. Chem.*, **213**, 557), that a histidine-requiring mutant of *A. aerogenes* could reach full growth in a medium containing glucose as the major source of carbon and a small supplement of histidine, in fact roughly

the amount of histidine required according to the histidine content of the bacterial protein. When glucose was replaced by another carbon compound, e.g. glycerol, inositol or pyruvate, about twenty times as much histidine had to be supplied to permit the culture to reach full growth. It was eventually found that glucose prevented the formation of the enzymes required for the conversion of histidine to glutamate, and thus preserved the histidine for its specific function, namely its incorporation into protein. The other carbon compounds were not metabolized as rapidly as glucose, and therefore failed to provide the repressor for the formation of the enzyme system responsible for the degradation of histidine; the conversion of histidine to glutamate, under these circumstances, provided the cell with additional energy and building blocks, but robbed it of histidine, the specific protein precursor.

In an organism capable of producing and of degrading histidine, the intracellular levels of histidine and of the as yet unidentified repressor of the histidine-degrading enzymes, together control the formation and subsequent fate of histidine. If the level of histidine is low, and that of the repressor is high, i.e. in the case of the organism growing in a glucose-containing medium without histidine, then histidine will be synthesized *de novo*, and not degraded, but used exclusively for protein synthesis. Conversely, if the level of histidine is high, and that of the repressor is low, i.e. in the case of the organism growing in a medium containing histidine, perhaps other carbon compounds, but no glucose, then histidine will not be synthesized *de novo* and the exogenous histidine will be used in part for protein synthesis, and in part degraded to provide additional energy and building blocks.

Prof. Potter spoke of the liver cell which fails to proliferate in a medium that presumably contains all the metabolites the cell requires for growth. He has suggested that the cell fails to grow because it destroys one or more of the essential metabolites. It would resemble in this case the histidine-requiring mutant of *A. aerogenes* in an environment containing a limited supply of histidine and a carbon and energy source other than glucose. In this condition, the intracellular level of the repressor is not high enough to prevent the formation of the histidine-degrading enzymes, and the histidine which is degraded cannot be replaced by synthesis *de novo*. Consequently, not enough histidine is available for protein synthesis, and the growth of the organism is restricted.

Lehninger: To change the trend of the discussion, are these instances described by Dr. Pardee and Dr. Magasanik just convenient rationalizations that can be made, in view of all these facts, or is there some conscious element of biological selection during

the course of evolution? If we were to take a series of reactions going from substrate A to product M, through *n* stages, I suspect that, during the course of evolution, at each reaction stage there might have been about ten different reaction possibilities taken from the organic chemistry textbook. We would end up ten reactions away with a compound, cytidylic acid, which can inhibit the first reaction. I have been mulling over the probability of this occurring during the course of selection. If there are ten different reaction possibilities at each of ten different reactions in sequence, this involves a lot of selection. Can Dr. Pardee and Dr. Magasanik say if there is a known genetical link between the cytidylic acid enzyme and the first one in the series? If there were a linkage this would be easier for me to understand. It would put two characters together, each of which is an end of a metabolic sequence.

Pardee: I don't think anyone has mapped the genes in pyrimidine synthesis.

Magasanik: In *Salmonella typhimurium* the genes responsible for histidine biosynthesis are closely linked and are arranged on the chromosome in the same order as the biosynthetic steps leading from AMP (or ATP) and ribose phosphate, the first specific step and the one inhibited by histidine, all the way to histidine (Hartman, P. E. (1957). *In* The Chemical Basis of Heredity, p. 408. Baltimore: Johns Hopkins Press; Moyed, H. S., and Magasanik, B. (1957). *J. Amer. chem. Soc.*, **79**, 4812; Moyed, H. S. (1958). *Fed. Proc.*, **17**, 279). The gene responsible for the reaction immediately preceding the first specific step in histidine biosynthesis, namely the gene controlling the enzyme which produces AMP from adenylosuccinate, is not linked to the "histidine genes". This finding would support your suggestion, Prof. Lehninger. However, as far as I know, the genes responsible for the synthesis of purines in the same organism are not closely linked; yet, there is good evidence that a derivative of guanine exerts a feedback effect by inhibiting an early step on the common pathway to guanine and adenine.

Lehninger: Speaking of sequential induction of a long series of enzymes in a chain, such as Stanier has done (Stanier, R. Y. (1947). *J. Bact.*, **54**, 339), are these all genetically linked?

Magasanik: No.

Lehninger: Would you say that the probability is fairly good?

Pardee: I do not think so. There is much argument among bacteriogeneticists as to whether sequences are genetically linked or not.

Lehninger: I can see the possibility of there having been some sense to the evolution of a system like this if all the enzymes in that sequence were adaptive. I am wondering about the constitutive

sequence, e.g. in glycolytic or respiratory enzymes which—in some organisms at least—are probably not adaptive, whether such a built-in feedback was selected or whether it was just pointed to by man with pride so that he could say "Here I can see the secret of life".

Potter: It was not selected. It was simply that if it had survival value it survived. That is all there is to it.

Lehninger: There is another and much simpler instance, i.e. the hexokinases: some are inhibited by glucose-6-phosphate and some are not. There must have been some element of survival value in one or the other kind of hexokinase.

Potter: In a particular constellation of enzymes.

McIlwain: A general point is that enzyme specificity as a whole will be subject to evolutionary control or selection: the question of what range of substrate will be affected by a given enzyme.

de Duve: If I understand Prof. Chance rightly he defined a controlling substance as a catalyst which can exist in two different states, and which functions in a cycle linking two processes together.

Chance: I do not think that the controlling substance is necessarily restricted to a catalyst; it can also be a coenzyme, provided it is in a form that can shuttle about.

de Duve: That is the point I was driving at. The controlling substances of most general importance, according to that definition, must be the coenzymes, because they happen to link the greatest amount of different reactions together. Another aspect of this is that the total amount of coenzyme acquires an important regulating function when it falls below optimum. If the amount of coenzyme decreases as the result, for instance, of an avitaminosis, then the competition between apoenzymes for this limited coenzyme becomes more and more important. The fact that reactions become deficient one after the other as the avitaminosis proceeds might be explained on this basis and one might surmise that the first enzyme to become defective will be the one with the lowest affinity for the coenzyme.

Chance: I would like to refer again to the discussion of Fig. IF (p. 360) in which we considered the possible coenzyme limitation of the citric acid cycle activity. Under such conditions, the enzyme system with the highest affinity for the coenzyme was already controlling the metabolism. In such a situation, a lowering of the coenzyme concentration would further enhance the control exerted by this reaction. If one considers the simultaneous function of pyridine nucleotide in the citric acid cycle and in glycolysis, then one system becomes deficient after the other, as you have pictured it.

Racker: Prof. Lynen has given us a stimulating discussion on the

problem of compartmentation in relation to glycogen synthesis in yeast (this symposium, p. 256). I wonder whether the calculations would still apply if the pathway of glycogen synthesis were different from the pathway of glycogen breakdown?

Lynen: The calculations would not apply only if the synthetic pathway were much more efficient than the degradative one. However, it was shown that an active phosphorylase exists in yeast cells. It does not matter whether this enzyme works in both processes or only in degradation. In either case, if there were no compartmentation the high orthophosphate/glucose-1-phosphate ratio would cause glycogen to be degraded.

Racker: If glucose-1-phosphate is not the intermediate in glycogen synthesis, but uridine diphosphate glucose is the true precursor, then the equilibrium calculation with inorganic phosphate as a reactant no longer applies.

Lynen: You would have a cycle where glycogen is broken down and synthesized again.

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Krebs: Before we disperse I would like to thank you for your enthusiasm and keenness which made the task of the Chairman easy.

I like to think that the symposium has been really worth while; worth while for those of you who have come a long way to spend a few days here, and worth while from the point of view of the Ciba Foundation. The subject of the symposium is bound to grow in importance in the coming years and I anticipate that the forthcoming book which records our discussions will be a very valuable one. As far as I am aware it will be the first book surveying a variety of aspects of the non-hormonal regulation of cellular metabolism and I hope it will prove to be a pioneer book.

AUTHOR INDEX

Plain numbers indicate a contribution by the author, either in the form of an article or to the discussions.
Numbers in italics indicate a reference to an author's work.

SUBJECT INDEX