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A HAEMOPEPTIDE FROM A TRYPTIC HYDROLYSATE OF *RHODOSPIRILLUM RUBRUM* CYTOCHROME *C**

By S. Paléus and H. Tuppy

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For the past few years the structure of cytochrome *c* has been studied with the aim of relating the catalytic activity of this haemoprotein to certain characteristic features of its chemical architecture. Much attention has been given to that portion of the protein moiety which adjoins the prosthetic group and which is supposed to contribute to a catalytically active site. In previous investigations (Tuppy and Bodo, 1954; Tuppy and Paléus, 1955) the sequence of twelve amino-acid residues, including two haem-bound cysteine residues, has been elucidated and found to be identical in the cytochromes of three different mammalian species (beef, horse and pig). When, however, the homologous amino-acid sequences were also determined in cytochrome *c* from fish (salmon), bird (chicken) (Tuppy and Paléus, 1955), insect (silk-worm) (Tuppy, 1957) and from a fungus (yeast) (Tuppy and Dus, 1958), they appeared to be dissimilar though resembling each other more or less closely. The present communication will extend this comparative structural investigation to the *c*-type cytochrome produced by the photosynthetic bacterium *Rhodospirillum rubrum*, first described and subsequently studied in more detail by Kamen and his co-workers (Vernon and Kamen, 1954; Kamen and Vernon, 1955; Bartsch and Kamen, 1958). There is a twofold interest in comparative structural work of this kind. In the first place, one may suppose that only those features which turn up invariably in cytochromes *c* from different sources are likely to be essential to the specific catalytic function, whereas structural differences will indicate points not directly concerned with catalytic activity. Secondly, the species similarities and dissimilarities as revealed by comparative investigations of amino-acid sequences may give a clue to the genetic relation between the organisms from which the proteins studied have been obtained.

---

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MATERIAL AND METHODS

Preparation of Rhodospirillum Rubrum *Cytochrome* c

Lyophilized bacterial cells were extracted with H_2SO_4 at pH 4. From the extract, the cytochrome *c* was obtained by fractionation with ammonium sulphate (Keilin and Hartree, 1937), dialysis and lyophilization. A sample thus prepared was kindly put at our disposal by Professor M. D. Kamen. It was further purified by chromatography on a column of CM-W cellulose (1·6 × 10 cm) prepared according to Peterson and Sober (1956) and buffered with 0·015 M ammonium acetate of pH 6·5. The preparation of cytochrome *c* (470 mg) was dissolved in 2 ml of this buffer, reduced by the addition of solid $Na_2S_2O_4$ and transferred to the column. A dark brown, fast moving band separated from the red cytochrome band which moved slowly, if at all. When the buffer of pH 6·5 was replaced by 0·05 M ammonium acetate of pH 7·5, the red material started moving down the column and divided into three bands. The main fraction ($R = 0{\cdot}4$, $E_{270}/E_{551} = 0{\cdot}96$) yielded 71·5 mg of dry, salt-free cytochrome *c* which was used for all subsequent experiments.

Preparation of the Haemopeptide and Removal of the Haem

After hydrolysis of the bacterial cytochrome *c* with trypsin, the haemopeptide formed could be precipitated from the digest with ammonium sulphate and purified by adsorption on talc and by column partition chromatography as described previously for other haemopeptides (Tuppy and Bodo, 1954; Tuppy and Paléus, 1955). The haemin part of the haemopeptide was split from the peptide moiety using silver sulphate in acetic acid solution according to the method of Paul (1949; 1950). Performic acid was used to oxidize the thiol groups of cysteine residues to sulphonic acid residues. The haemin-free oxidized peptide obtained was essentially homogeneous as determined by electrophoresis on paper in a formic acid–acetic acid buffer of pH 2 (Werner and Westphal, 1955).

Enzymic Hydrolyses

The crystalline chymotrypsin used was purchased from the Worthington Biochemical Corporation. The subtilisin was a gift from Ing. M. Ottesen, Carlsberg Laboratory, Copenhagen. Digestions were carried out at pH 7·5 to 7·8 for 20 hr at 25°C (with chymotrypsin) or for 24 hr at 37°C (with subtilisin), the ratio between enzyme and substrate (w/w) being about 1:15.

End Group Determinations

For the characterization of N-terminal amino-acid residues the DNP method (Sanger, 1945; see also Sanger and Thompson, 1953) was employed. C-terminal residues were determined using the hydrazinolysis procedure of Akabori, Ohno and Narita (1952).

RESULTS

In the hydrolysate of the haemin-free oxidized polypeptide (prepared from the haemopeptide as described in the foregoing section) ten different amino-acids were identified by paper chromatography—alanine, aspartic acid, cysteic acid, glutamic acid, glycine, histidine, leucine, lysine, phenylalanine and threonine. The lysine present in the polypeptide may be expected to be the C-terminal residue of the amino-acid sequence, since the polypeptide has been formed by the action of trypsin which is known to split bonds involving the carboxyl groups of lysine and arginine residues. The N-terminal residue was shown to be cysteic acid. After dinitrophenylation of the polypeptide and subsequent hydrolysis, however, the hydrolysate was found to contain, besides DNP-cysteic acid, free cysteic acid as well. This finding is accounted for by the presence in the polypeptide of two cysteic acid residues, one of which is terminal and the other is not.

Chymotryptic hydrolysis of the polypeptide (Table 1) gave rise to three main split products which were separated from each other by high-voltage electrophoresis at pH 2 and by paper chromatography in butanol–acetic acid: (I) $CySO_3H$–[Leu, Ala, $CySO_3H$, His], (II) Thr–Phe, and (III) Asp–Glu–[Gly, Ala, Asp]–Lys. Split product (I) with N-terminal cysteic acid, must be derived from the N-terminal side of the polypeptide chain and the lysine-containing peptide (III) from the C-terminal side. Thr–Phe, then, must be located between these two. After dinitrophenylation of peptide III and partial hydrolysis (with conc. HCl at 37°C, for 8 days) two ether-soluble products were separated and characterized, DNP–Asp and DNP–Asp–Glu, which demonstrates that glutamic acid must adjoin the N-terminal aspartic acid residue.

In the subtilisin hydrolysate of the polypeptide (Table 1) five products were present in relatively high concentration: $CySO_3H$–Leu–Ala, $CySO_3H$–His, Thr–Phe, [Asp, Glu, Gly, Ala], and Asp(NH_2)–Lys. Among the split products obtained in small amounts there were two tripeptides containing histidine: Ala–[$CySO_3H$, His] and $CySO_3H$–[His, Thr]. Other minor components of the hydrolysate were shown to be [Gly, Ala], [Asp, Glu, Gly], [Thr, Phe, Asp, Glu, Gly] and free alanine.

From the findings presented above it may be concluded that the amino acid sequence in the haemin-free oxidized polypeptide is

$$\overset{1}{CySO_3H}\text{—}\overset{2}{\text{Leu}}\text{—}\overset{3}{\text{Ala}}\text{—}\overset{4}{CySO_3H}\text{—}\overset{5}{\text{His}}\text{—}\overset{6}{\text{Thr}}\text{—}\overset{7}{\text{Phe}}\text{—}\overset{8}{\text{Asp}}\text{—}\overset{9}{\text{Glu}}\text{—}\overset{10}{\text{Gly}}\text{—}\overset{11}{\text{Ala}}\text{—}\overset{12}{\text{Asp}(NH_2)}\text{—}\overset{13}{\text{Lys}}$$

The results obtained leave it undecided whether residues 8 and 9 are free aspartic acid and free glutamic acid or whether these amino acids are in the amide form, as asparagine and glutamine residues.

The two cysteic acid residues contained in the sequence have arisen from and taken the place of two haem-bound cysteine residues present in the

Table 1. Determination of the amino acid sequence in a polypeptide obtained from *Rhodospirillum rubrum* cytochrome *c* by tryptic hydrolysis, removal of the haem moiety, and oxidation with performic acid

N-terminal residue (DPN method)		$CySO_3H$— . . .
C-terminal residue (specificity of trypsin)		. . . —Lys
Peptides obtained by *chymotryptic* digestion		$CySO_3H$—[Leu, Ala, $CySO_3H$, His] Asp—Glu—[Gly, Ala, Asp]—Lys Thr—Phe
Peptides found after digestion with *subtilisin*	Main split products	$CySO_3H$—Leu—Ala Thr—Phe $\overset{\mathrm{NH_2}}{\overset{\vert}{\mathrm{Asp}}}$—Lys $CySO_3H$—His [Asp, Glu, Gly, Ala]
	Minor split products	Ala—[$CySO_3H$, His] [Asp, Glu, Gly] [Thr, Phe, Asp, Glu, Gly] $CySO_3H$—[His, Thr] [Gly, Ala]
Sequence		$CySO_3H$—Leu—Ala—$CySO_3H$—His—Thr—Phe—Asp—Glu—Gly—Ala—$\overset{\mathrm{NH_2}}{\overset{\vert}{\mathrm{Asp}}}$—Lys

TABLE 2. COMPARISON OF THE SEQUENCES OF AMINO ACIDS IN THE VICINITY OF THE PROSTHETIC GROUP IN *c*-TYPE CYTOCHROMES OF DIFFERENT ORGANISMS

Ox Horse Pig	$\ldots\text{—Val—}\overset{\substack{\mathrm{NH_2}\\ \vert}}{\mathrm{Glu}}\text{—Lys—CyS—Ala—}\overset{\substack{\mathrm{NH_2}\\ \vert}}{\mathrm{Glu}}\text{—CyS—His—Thr—Val—Glu—Lys—}\ldots$
Salmon	$\ldots\text{—Val—}\overset{\substack{\mathrm{NH_2}\\ \vert}}{\mathrm{Glu}}\text{—Lys—CyS—Ala—}\overset{\substack{\mathrm{NH_2}\\ \vert}}{\mathrm{Glu}}\text{—CyS—His—Thr—Val—Glu—}\ldots$
Chicken	$\ldots\text{—Val—}\overset{\substack{\mathrm{NH_2}\\ \vert}}{\mathrm{Glu}}\text{—Lys—CyS—Ser—}\overset{\substack{\mathrm{NH_2}\\ \vert}}{\mathrm{Glu}}\text{—CyS—His—Thr—Val—Glu—}\ldots$
Silk-worm	$\ldots\text{—Val—}\overset{\substack{\mathrm{NH_2}\\ \vert}}{\mathrm{Glu}}\text{—Arg—CyS—Ala—}\overset{\substack{\mathrm{NH_2}\\ \vert}}{\mathrm{Glu}}\text{—CyS—His—Thr—Val—Glu—}\ldots$
Yeast	$\ldots\text{—Phe—Lys—Thr—Arg—CyS—Glu—Leu—CyS—His—Thr—Val—Glu—}\ldots$
Rhodospirillum rubrum	$\ldots\text{—CyS—Leu—Ala—CyS—His—Thr—Phe—Asp—Glu—Gly—Ala—}\overset{\substack{\mathrm{NH_2}\\ \vert}}{\mathrm{Asp}}\text{—Lys—}\ldots$

original *Rhodospirillum rubrum* cytochrome *c* and in the haemopeptide prepared from it by tryptic hydrolysis.

The specificity of trypsin already mentioned suggests that the amino-acid residue preceding the above sequence must be either lysine or arginine.

The cleavage by chymotrypsin of a bond between histidine and threonine (residues 5 and 6) may appear unexpected in view of the general belief that chymotrypsin will split bonds involving the carboxyl groups of the aromatic residues phenylalanine and tyrosine. The finding is in agreement with recent observations of Davis (1956), who, however, has reported that bonds involving the carboxyl groups of histidine residues are somewhat susceptible to chymotryptic hydrolysis.

The comparison of the amino-acid sequences that have been shown to occur in the vicinity of the haem moiety in different cytochromes demonstrates that they have various structural features in common (Table 2). The presence of two cysteine residues linked to side chains 2 and 4 of the porphyrin of the prosthetic group, first indicated by Theorell (1939) for ox cytochrome *c*, has been confirmed and found to be characteristic of all *c*-type cytochromes. The distance between the two cysteine residues along the polypeptide chain is always the same, two other amino acids being situated in between. Invariably a histidine residue has been found to adjoin one of the haem-bound cysteine residues. Stereochemical considerations (Ehrenberg and Theorell, 1955) have indicated that the chain of amino acids in which the two haem-bound cysteine residues and the histidine residue are included is likely to be in the form of an α-helix. This coiling will bring the histidine residue into a steric position favourable for covalent attachment of an imidazole nitrogen to the iron of the haem disc.

There are still other structural features encountered in all cytochrome *c* samples examined so far, such as, for example, the presence of a basic residue (lysine or arginine) close to cysteine and of a threonine residue close to histidine. The significance of these similarities is at present not known.

It is remarkable that in so many structural respects the cytochrome *c* of *Rhodospirillum rubrum* appears to resemble the *c*-type cytochromes of other organisms, in spite of an overall amino-acid composition, oxidation-reduction potential, and a specificity of action quite different from those of vertebrate and yeast cytochromes (Kamen and Takeda, 1956).

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ELECTROMETRIC AND OTHER STUDIES ON CYTOCHROMES OF THE *C*-GROUP

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THERE is a very wide distribution in tissues, of pigments with a haemochrome-type spectrum showing absorption peaks in the visible region close to 550 and 520 mμ. A number of haemochromes with different haem prosthetic groups are known, however, to show a similar spectrum (see for example, Lemberg and Legge, 1949; Morton, 1958) and from time to time it has been pointed out that many of these compounds are as yet only spectral phenomena. With the isolation of bacterial cytochromes (see for example, Kamen

TABLE 1. RANGE OF E_0' VALUES FOUND IN CYTOCHROME *c* GROUP PIGMENTS (see also Morton, 1958)

Common symbol	Source	E_0' (V)	
f	Plants (chloroplasts)	+0·365	Davenport & Hill (1952)
c_2	*Rhodospirillum rubrum*	+0·338	Kamen & Vernon (1955)
c	Mammalian heart-muscle	+0·255	Rodkey & Ball (1950) Henderson & Rawlinson (1956a)
c	*Micrococcus denitrificans*	+0·250	Kamen & Vernon (1955)
RHP-pigment	*Rhodospirillum rubrum*	−0·008	Bartsch & Kamen (1958)
c_3	*Desulphovibrio desulphuricans*	−0·205	Postgate (1956)

TABLE 2. E_0' VALUES OBTAINED AFTER MODIFICATION OF MAMMALIAN HEART-MUSCLE CYTOCHROME *c*

Treatment	E_0' (V)	
Fraction separated on a resin column from TCA-extracted preps.	+0·310	Henderson & Rawlinson (1956b)
'Resin-purified' prep. treated with 2·5% TCA for 18 hr, 25°C	+0·308	Henderson & Rawlinson (1956b)
Proteinase-digested	−0·3	Minakami, Titani & Ishikura (1957)

cf. Table 1, resin-purified cytochrome *c*, $E_0' = +0{\cdot}255$ V.

and Vernon, 1955) and the modification of mammalian heart muscle cytochrome *c* by treatments such as proteolytic digestion (Tsou, 1951a) many varied properties are apparent in pigments having the same or closely similar absorption spectra.

This variation is observed in the important property of oxidation-reduction potential (E_0') even where the haem prosthetic groups are known to be of the mammalian heart cytochrome *c* (M. heart *c*) type (Table 1). It is particularly interesting that a difference in E_0' at least as large as that existing between the naturally occurring '*c*-type' pigments may be obtained by modification of M. heart *c* without any significant spectral change (Table 2).

OXIDATION-REDUCTION POTENTIAL OF YEAST CYTOCHROME *C*

The early controversy regarding the E_0' value of yeast cytochrome *c* centred around the values of +0·26 V (Coolidge, 1932) and +0·12 V (Green, 1934) (see also Table 3). It was generally considered to have been resolved in favour of the former by what is now obviously the doubtful procedure of comparing the values already obtained from yeast samples with those from several other sources, especially mammalian heart muscle. Furthermore, Green's criticisms of Coolidge's methods seem quite valid. The problem does not appear to have been attacked again using the isolated pigment until Minakami (1955) reported some potential levels for several ratios of oxidized/reduced cytochrome *c* of yeast which were similar to those for M. heart *c*. It still seemed desirable to carry out a complete electrometric titration of yeast cytochrome *c* extracted under mild conditions and avoiding the use of $Na_2S_2O_4$ as titrant (see, for example, Paul, 1951). This was done using a sample (kindly provided by Prof. R. K. Morton) which had been extracted from dried baker's yeast with NaCl and chromatographed on a resin exchange

TABLE 3. E_0' VALUES OF VARIOUS YEAST CYTOCHROME *c* SAMPLES

Extraction method	E_0' (V)	pH	Reference
(i) Ammonium sulphate extract (ii) Cells plasmolysed at boil then treated with SO_2 (iii) Whole cell suspension	+0·26	7	Coolidge (1932)
SO_2 method of Keilin (1930)	+0·123	7	Green (1934)
Plasmolysed (boiled) cell suspension	+0·12 to +0·16	7	de Toeuf (1937)
Washed yeast cell suspension	+0·11 to +0·15	6·8	Baumberger (1939)
SO_2 method of Keilin (1930) then resin (XE–64) treatment	+0·26 to +0·27	6, 7	Minakami (1955)

column (Armstrong, Coates and Morton, 1958, and this volume, p. 385). The value of +0·28 V with $n = 1$ at pH 6·4 and 25°C was obtained (Fig. 1; see also Henderson and Rawlinson, 1958).

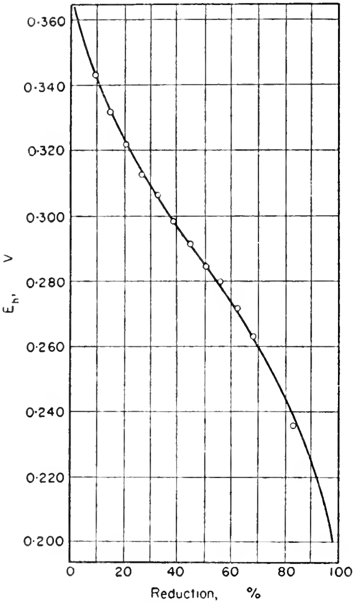

FIG. 1. Oxidation-reduction titration at pH 6·4 of yeast cytochrome *c*. Solid line is theoretical curve for $E_0' = 0{\cdot}282$ V, $n = 1$.

COMBINATION OF CYTOCHROME *C* FRACTIONS WITH COPPER

Inhibition of Copper-catalysed Oxidation of Ascorbic Acid

One of the most striking effects of proteolytic digestion of M. heart *c* is the induction of a high ascorbic acid oxidase activity which parallels a loss in activity in the usual biological oxidase systems (Tsou, 1951b). Margoliash (1954b) observed a similar effect with a fraction separated on a resin column.

When examining the ascorbic acid oxidase activity of a number of M. heart *c* fractions we observed that in the case of the '0·34% Fe' or 'low-iron' content samples, lower oxygen-uptake values were obtained than for the ascorbate blank. A similar reduction in oxygen-uptake of the blank was obtained by addition of ethylenediaminetetraacetic acid (EDTA) (Fig. 2).

The copper catalysis of ascorbate oxidation is well known (see, for example, Barron, de Meio and Klemperer, 1935) and up to about 10^{-8} g atom/ml the rate of oxidation is directly proportional to the concentration of copper. It seemed likely that the '0·34% Fe' content samples were inhibiting an oxidation of ascorbate catalysed by traces of copper present as an impurity in the reagents. A series was therefore run in which there was a constant added concentration of copper and varying amounts of the different cytochrome *c* fractions (Fig. 2). Three distinct types of curves were obtained. The '0·34% Fe' cytochrome *c* gave a value the same as in the absence of added copper until the point corresponding to approximately 4 cytochrome *c* molecules/copper atom, after which there was a rapid rise in uptake inversely proportional to the cytochrome concentration. The '0·43% Fe' cytochrome *c* inhibited the ascorbate oxidation to a much lesser extent than the above material. This indicated that combination was in the main occurring between the copper and a colourless protein fraction which could be removed electrophoretically (Theorell and Åkeson, 1941) or chromatographically (Paléus

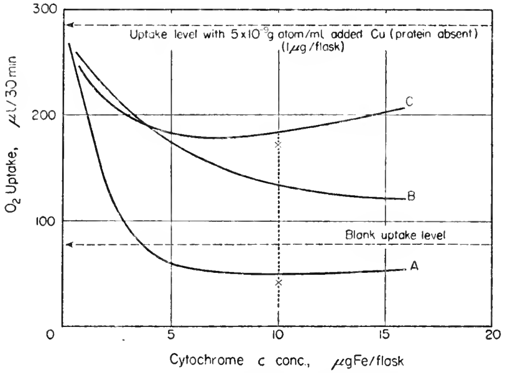

FIG. 2. Influence of cytochrome *c* fractions on ascorbate oxidation. (Determinations using Warburg manometers, 0·07 M sodium phosphate buffer, pH 7·3 and 0·02 M sodium ascorbate, at 37°C.)

I. *With added copper* (1 *μg/flask*).

Curve *A*: Preparations: (i) before resin column treatment, and (ii) colourless protein fraction separated electrophoretically.
Curve *B*: Preparations: (iii) after resin column treatment, and (iv) after electrophoresis, and (v) after treatment with TCA.
Curve *C*: (vi) cytochrome fraction separated on resin column.

II. *No copper added.*

Fractions (i) to (v) inclusive and also 10^{-3} M EDTA lowered the oxygen uptake to the bottom *X*; fraction (vi) catalysed the oxygen-uptake to the top *X*.

and Neilands, 1950; Margoliash, 1954a, b). Presumably the same material is removed when the iron content is increased by the alkaline salt fractionation of Keilin and Hartree (1945) or the boiling $CHCl_3$ treatment of Tsou (1951a). This colourless fraction was accordingly separated in the Tiselius-type electrophoresis apparatus and found to combine with copper producing a curve of type *A*, Fig. 2.

Zinc Precipitation of Myoglobin

The question arises as to whether the colourless protein fraction is present *in vivo*. Margoliash (1954a) considered it to be globin present as an artefact of preparation due to splitting of myoglobin under the conditions of the extraction with trichloroacetic acid (TCA). In the case of neutral extraction procedures some myoglobin was present. In order to examine further this question, cytochrome *c* was extracted from heart muscle with saline solution. The resulting solution contained the pigments haemoglobin (Hb) and myoglobin (Mb) in addition to cytochrome *c*. It had been shown by one of us (Rawlinson, 1938) that Hb and globin may be quantitatively precipitated by Zn^{++} at slightly alkaline pH. Myoglobin was accordingly tested under similar conditions and found to behave in the same manner. Haemoglobin and myoglobin along with much colourless protein material were therefore removed from the saline extract of cytochrome *c* by this method. Up to this point, the conditions had been such that no splitting of the prosthetic

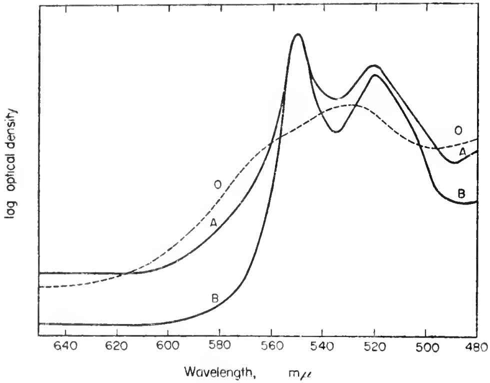

FIG. 3. Comparison of characteristic absorption spectra of oxidized and reduced cytochrome *c* samples from ox-heart muscle. Curve *A*: salt-extracted and Zn-treated. Curve *B*: the former after resin treatment. Curve *O*: the oxidized forms of both samples.

groups from Hb or Mb would be expected. The cytochrome *c* was then concentrated with TCA as in the final stage of the Keilin and Hartree procedure. This material had an iron content of 0·32% and a trace only of zinc was present. Prior to TCA-treatment no absorption at 630–635 mμ due to metmyoglobin or methaemoglobin could be detected and subsequently the absorption spectra of the oxidized forms before and after resin chromatography were identical (Fig. 3). No fore-running non-cytochrome fraction containing protohaem could be detected on resin column chromatography as is the case with TCA-extracted samples. However, increased absorption, particularly on the longer wave-length slope of the α-band is apparent in the reduced form of the material prior to resin treatment (Fig. 3) and the band ratios differ considerably (Table 4). Inhibition of the copper-catalysed oxidation of ascorbic acid was similar to that of the usual '0·34% Fe' content preparations, indicating combination with copper. A sample which had been treated with copper under the conditions described in Fig. 2, was given a repetition of the final TCA precipitation of the Keilin and Hartree procedure. It was found that this material would then again take up a similar quantity of copper. It appeared therefore, as in fact would be expected, that the copper originally combined was removed under the acid conditions.

TABLE 4. COMPARISON OF OPTICAL-DENSITY RATIOS OF SEVERAL OX-HEART CYTOCHROME *c* SAMPLES

Extraction method	% Fe	*Ratio of* $\frac{\text{590 m}\mu\text{ (oxidized)}}{\text{590 m}\mu\text{ (reduced)}}$	*Ratio of* $\frac{\text{550 m}\mu\text{ (reduced)}}{\text{520 m}\mu\text{ (reduced)}}$	*Ratio of* $\frac{\text{550 m}\mu\text{ (reduced)}}{\text{535 m}\mu\text{ (reduced)}}$	*Ratio of* $\frac{\text{520 m}\mu\text{ (reduced)}}{\text{535 m}\mu\text{ (reduced)}}$
(a) Saline (Zn-treated)	0·32	1·95	1·55	2·53	1·63
(b) TCA (Keilin and Hartree method)	0·30	1·93	1·74	3·38	1·94
(c) (a) After single resin column, pH 7 (Margoliash, 1954b)	—	3·13	1·79	3·92	2·20
(d) (b) After double resin column, pH 7 and 9·6 (Margoliash, 1954b)	0·44	5·78	1·76	3·86	2·19

Combination of Copper with Globin

Myoglobin was prepared from horse-heart by a combination of the methods of Theorell (1932) and Lawrie (1951) and globin from this by the procedure of Theorell and Åkeson (1955). Globin was added in varying concentration to constant copper and ascorbate concentration as for the cytochrome *c* fractions (Fig. 2) and the O_2 uptake determined (Fig. 4). Great avidity for copper was shown; combination, however, was in the ratio of 2 g atom of Cu/mole globin.

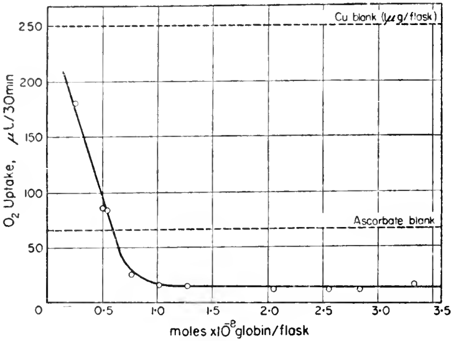

FIG. 4. Inhibition by globin (from horse-heart myoglobin) of the copper-catalysed oxidation of ascorbate. (Experimental conditions: Warburg manometers; 0·07 M sodium phosphate buffer, pH 7·3; 0·02 M sodium ascorbate, and 1 μg of copper/flask at 37°C.)

E_0' of Cytochrome c *in the Presence of Copper*

Low-iron content samples in general show a slightly higher E_0' (about 15 mV) than the higher-iron content samples (Henderson and Rawlinson, 1956a). This was attributed, however, to the presence of modified cytochrome *c*. We have now examined the E_0' of a '0·34% Fe' content sample of M. heart *c* before and after addition of copper in slight excess of its binding capacity and find the value to remain unaltered ($E_0' = + 0{\cdot}26$ V, $n = 1$). The sample containing copper showed a somewhat increased rate of autoxidation. This electrometric result is compatible with an aggregation where there is no significant interaction one with another between the groups oxidized/reduced (Shack and Clark, 1947), although of course it neither proves nor disproves a state of aggregation.

Crystallization of Cytochrome c *from a 'Low-iron' Content Preparation*

Since the first crystallization of cytochrome *c* from King penguin muscle by Bodo (1955) there have been several reports, notably from the school of Okunuki, of crystallization of the pigment from a number of different tissues (see Morton, 1958). Much work has been carried out on cytochrome *c* from mammalian heart muscle, especially from ox-heart. To the best of our knowledge cytochrome *c* from this source had been crystallized only after resin-column treatment.

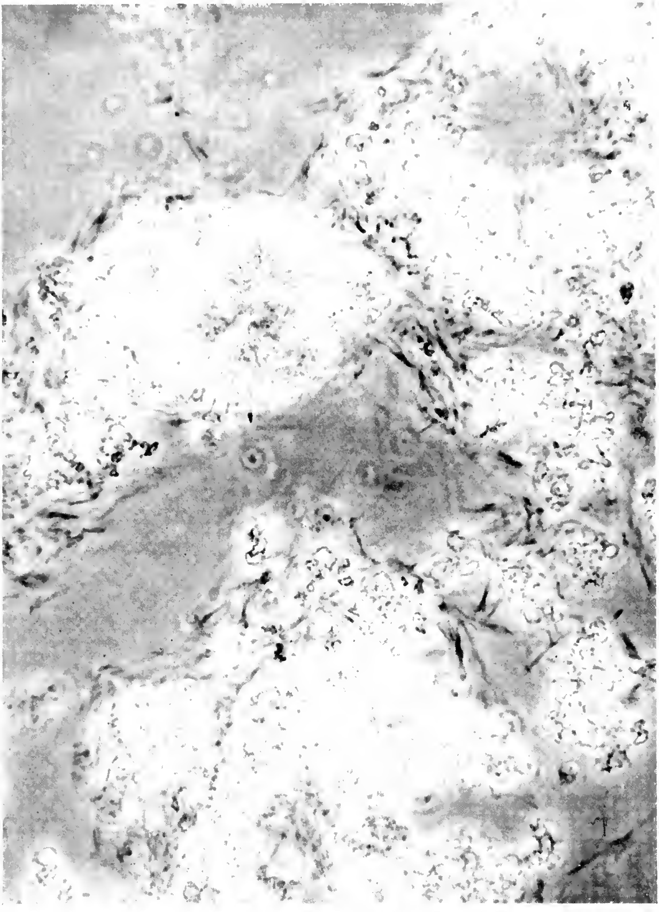

FIG. 5. Cytochrome *c* crystals after 48 hr, phase contrast, ×800.

Recently, however, we have been successful in crystallizing the pigment from a '0·31% Fe' content sample obtained simply by the usual Keilin and Hartree procedure. The crystallization method of Hagihara, Morikawa, Tagawa and Okunuki (1958) was followed except that an addition of copper was made in the binding ratio of 1 g atom Cu/4 moles cytochrome *c* (see Henderson and Rawlinson, 1960). Small crystals formed rapidly (1–2 hr) and within 48 hr many rosettes were present along with crystals (Fig. 5) having an appearance very similar to those depicted by Hagihara *et al.* (1956) and described by them as leaflets. The crystals were in the reduced form. The biological activity and other properties of this preparation have not yet been investigated.

Possible Linkage of Cytochrome c in vivo

The above experiments show that a colourless protein fraction is associated with M. cytochrome *c* extracted both with and without the use of acid, and in the latter case when myoglobin and haemoglobin would appear to have been completely removed. This fraction has an anodic mobility at pH 7·4 and from this fact it is to be expected that some affinity exists between this material and M. cytochrome *c* with its high iso-electric point (pH 10·5). There is some evidence that the fraction is globin but from the above experiments it would seem that there is still room for doubt.

The view has been put forward previously that this material is associated with cytochrome *c in vivo* (see Lemberg and Legge, 1949). This view was based largely on the behaviour of '0·34% Fe' cytochrome *c* with regard to such properties as heat stability and difficulty of separation except by electrophoresis. If it is in fact present *in vivo* it may be that the strong metal-binding capacity shown above is the means of bringing about the particular spatial or other arrangement—even of electron transport—which it seems is necessary (see Slater, 1958) for full endogenous activity.

'Modified' Cytochrome c

After separation of the main fraction of cytochrome *c* on a resin column (Fraction I, pH 7 according to Margoliash, 1954b), there is left a band of pigment at the top of the column. This material (Fraction II) may be eluted with 0·5 N NH_4OH. Margoliash (1954b) found it to have an increased ascorbic acid oxidase activity and a lowered biological activity. Some of the colourless protein fraction is, however, eluted at the same time by the NH_4OH. After further chromatography to remove as much as possible of the latter, a relatively high ascorbic acid oxidase activity resulted (curve *C*, Fig. 2).

The E_0' value of this fraction has already been mentioned (Table 2) to be some 55 mV above that of the main fraction. It also seems very likely that the state of aggregation is altered, as it is seen from Fig. 6 that the value of

n which is between 1 and 2 at the commencement of titration, approaches 2 as the reduction continues, and after the point of 50% reduction is quite close to 2. These changes would appear to provide an explanation of the altered biological activity of this fraction.

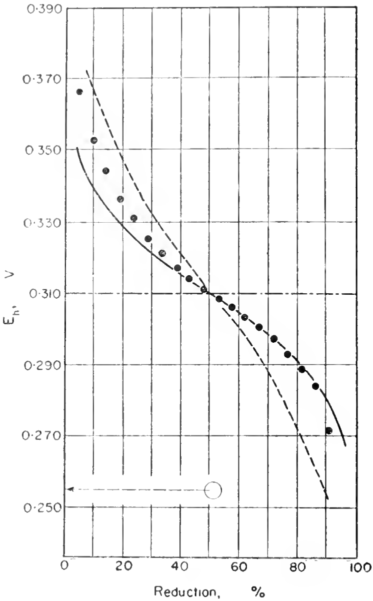

FIG. 6. Oxidation-reduction titration at pH 6·4 of 'modified' ox-heart cytochrome c (Fraction II according to Margoliash, 1954b). Observed points ●; theoretical curves are for $E_0' = +0{\cdot}310$ V; broken line, $n = 1$; solid line, $n = 2$. The oxidation-reduction potential of 'unmodified' fraction ($E_0' = +0{\cdot}255$ V) is shown thus ○.

Margoliash (1954a, b) obtained evidence that in the case of horse-heart cytochrome c, TCA at pH 4·5 was responsible for the formation of Fraction II above. We subjected resin-purified samples from ox-heart to treatment with TCA at approx. pH 1, (2·5% TCA, 18 hr, 25°C) and obtained a similar E_0' value to that of Fraction II. Again there was evidence of aggregation although in this case it was not as pronounced as in Fraction II above; the curve was in between $n = 1$ and $n = 2$ after 50% reduction. It is of considerable interest that the ascorbic acid oxidase activity remained virtually unaltered (Fig. 2). It did not behave on the resin column like Fraction II as it was eluted slowly and diffusely by 0·25 M ammonium acetate at pH 7.

From one such TCA-treated cytochrome *c* sample a small residue was obtained after dialysis against 0·5% NaCl which dissolved in 0·02 N NH_4OH. This material had an increased ascorbic acid oxidase activity and a somewhat altered absorption spectrum (Fig. 7). This result was variable, however, and generally no residue was obtained. There was thus evidence for the production of more than one fraction by this treatment, which is in agreement with the observations of Margoliash, Frohwirt and Wiener (1959) who have reported a separation of Fraction II into several other fractions with varying properties.

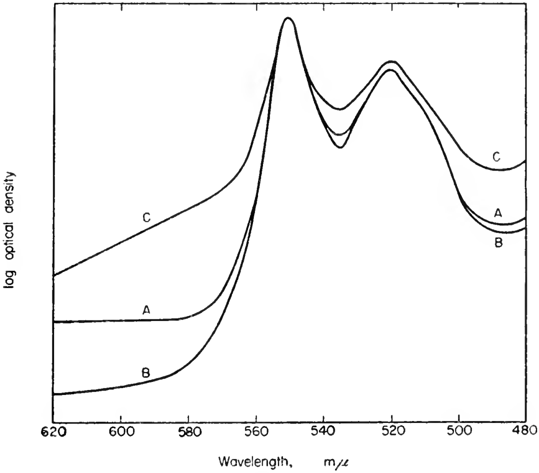

FIG. 7. Comparison of characteristic absorption spectra of reduced cytochrome *c* samples from ox-heart muscle. Curve *A*: resin-treated sample; curve *B*: fraction soluble in 0·5% NaCl after 2·5% TCA, for 18 hr, 25°C; curve *C*: residue after TCA treatment, soluble in 0·02 N NH_4OH.

Although TCA under various conditions can bring about changes in cytochrome *c* such as increased susceptibility to proteinase digestion, change in chromatographic behaviour and altered E_0', it seems that with mild treatment its biological activity is unaltered (see, for example, Keilin and Hartree, 1955). In fact the modified material—as judged by its chromatographic behaviour—may show unchanged properties in biological systems (see, for example, Hagihara *et al.*, 1958a; Yamanaka *et al.*, 1959).

In connexion with the often-quoted autoxidation and combination with CO as criteria of modification, it is noteworthy that Minakami *et al.* (1958), after acetylation of the ε-lysine residues, obtained a product inactive in the succinic oxidase system and autoxidizable but which showed neither ascorbic acid oxidase activity nor combination with CO. Furthermore, guanidation of these residues (Takahashi *et al.*, 1958) left the pigment practically unaltered in relation to these properties. These findings along with those of Nozaki *et al.* (1957, 1958), who showed dependence of resistance to proteolytic attack on the state of oxidation of the pigment, seem to be, as postulated by these workers, an indication of the involvement of the secondary protein structure in the oxidation-reduction reactions of cytochrome *c*.

E_0' CHANGE WITHIN THE CYTOCHROME *C* GROUP

An explanation for the variation in E_0' values quoted in Tables 1 and 2 may be found in terms of the following general equation derived by Clark *et al.* (1940). This relates the E_0' of a metal co-ordinate complex (of the type with which we are dealing here) to change in degree of association of the ligand with the central metal atom.

An outline only of the derivation is given below, and reference should be made to the original for full details of assumptions.

From the basic equation—

$$O_n + ne = pR_m \qquad (1)$$

where O_n and R_m are the oxidant and reductant respectively in the fixed states of aggregation designated by n and m, the usual electrode equation may be derived:

$$E_h = E_o + \frac{RT}{nF} \ln \frac{(O_n)}{(R_m)^p} \qquad (2)$$

Co-ordination is then assumed to occur with a nitrogenous base B such that there is no change in the respective states of aggregation; then:

$$O_n + qB = O_nB_q \qquad (3)$$

$$R_m + rB = R_mB_r \qquad (4)$$

from which the respective equilibrium constants K_O and K_R may be obtained.

Under the conditions $p = m = n = 1$ and at 30°C, the following equation may be obtained:

$$E_h = E_o + 0{\cdot}06 \log \frac{S_o}{S_r} + 0{\cdot}06 \log \frac{K_O}{K_R} + 0{\cdot}06 \log \frac{K_R + [B]^r}{K_O + [B]^q} \qquad (5)$$

where $S_o = n(O_n) + n(O_nB_q)$

and $S_r = m(R_m) + m(R_mB_r)$

It can be seen from equation (5) that, other things being equal, E_h is dependent upon the values K_O and K_R, i.e. the E_0' value is dependent upon these two constants.

In the cytochrome *c* group, the ligands to the 5th and 6th co-ordinate positions are attached to the apo-protein. It is conceivable that owing to differences between the natural apo-protein structures or to modifications which bring about perhaps some unfolding of these structures, there is variation in the strain and in consequence of the stability of the ligand bondings. In such cases the resultant alterations in values of K_O and K_R would be reflected in the E_0' values.

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DISCUSSION

Protein Configuration and Linkage to the Prosthetic Group in Cytochrome c

Studies of the Haemochrome-forming Groups in Cytochrome *c*

By E. MARGOLIASH (Utah)

MARGOLIASH: For all the typical haemochrome properties there is no sharp breaking point between native cytochrome *c* on the one hand and the pepsin digested cytochrome *c* 'core', which can be considered as the most degraded form of the cytochrome *c* haemochrome, on the other. Quite the contrary, by isolating by column chromatography a series of cytochromes *c* showing increasing degrees of denaturation it could be shown that all the haemochrome properties vary gradually with the degree of denaturation. These fractions of denatured cytochrome *c* were defined by their chromatographic properties on Amberlite IRC–50, the more protein being denatured the larger the number of ε-NH_2 groups of lysine available to the resin and for reaction with a reagent such as 1-fluoro-2-nitro-4-benzene sulphonate.

Native cytochrome *c* was at one end of the scale with maximal enzymic activity in the succinic oxidase and cytochrome oxidase systems, a very low rate of auto-oxidation, a small per cent combination with CO at near neutral pH values, and a very wide pH-range of stability of the reduced spectrum. With increasing degrees of denaturation the enzymic activities became lower, the rate of auto-oxidation and the per cent combination with CO increased; the pH-range of stability of the reduced spectrum became gradually smaller moving toward the alkaline side. With respect to all these properties the cytochrome *c* 'core' is in every way identical to the most denatured form of cytochrome *c* obtained, even though it contains only 11 amino acids out of the full complement of 96–98 present in denatured cytochrome *c*.

The cytochrome *c* 'core' shows on reduction the typical cytochrome *c* haemochrome spectrum, but in contradistinction to native cytochrome *c*, the reduced spectrum of which is completely unaffected by pH over a very wide range from pH 2–3 to pH 13, the 'core' acts like a normal chemical haemochrome. Measuring at the maximum of the α band, one finds that the spectrum of the 'core' reaches the full extinction of native cytochrome *c* only at about pH 11 after passing through 3 sets of inflexions with mid-points at 5·4, 7·6 and 9·5. The addition of a large excess of bases containing α- or ε-NH_2 groups did not effect the maximal extinctions obtained at pH 11, whereas the addition of bases containing only an imidazole group decreased the height of the α-band to 5–8% below that of native cytochrome *c*. This was interpreted as indicating that the haemochrome-forming groups in cytochrome *c* are not both imidazoles. We therefore investigated the possibility that the ε-NH_2 group of the lysine and the imidazole group of the histidine, both amino acids being the residues adjacent to the cysteines involved in the thio-ether bonds holding the haem to the protein, were the actual haemochrome-forming groups in cytochrome *c*.

Ehrenberg and Theorell (*Acta chem. Scand.* **9**, 1193, 1955) had shown that if the peptide chain of the 'core' were made into an α-helix either the imidazole or the ε-NH_2 group could be made to coordinate with the central haem iron atom, but not both at the same time. However, building a suitable model it could readily be shown that if the intervening chain of 4 amino acids were extended, these two groups could indeed be made to occupy coordination positions 5 and 6 of the iron atom, above and below the plane of the haem.

A study of the ultracentrifugal sedimentation characteristics of the 'core' indicated that although the 'core' was probably a small polymer at intermediate pH values, at those alkaline pH values at which its spectrum maximally approached that of native cytochrome *c* the 'core' was a monomer and the haemochrome must necessarily have been intramolecular, rather than intermolecular as could have been the case for a polymer.

These studies (*Biochem. J.* **71**, 559, 1959) have led us to conclude that: (a) the haemochrome-forming groups in cytochrome *c* are probably the ε-amino group of the lysine in position 3 and the imidazole group of the histidine in position 8 of the amino-acid sequence of the pepsin-digested cytochrome *c* 'core' (see Paléus' paper); (b) the properties particular to the haemochrome of native cytochrome *c*, as contrasted with those of a normal chemical haemochrome, appear to be determined by the effect of the entire protein in its native configuration on the iron-ligand bonds, rather than by the particular chemical groups involved in the haemochrome of cytochrome *c*.

The Amino-acid Sequence in Horse-heart Cytochrome *c*

By E. Margoliash and R. Hill (Utah)

Margoliash: I should like to report on two lines of work dealing with the amino-acid sequence of horse-heart cytochrome *c*.

(i) *Leucine aminopeptidase digestion of cytochrome* c. Using a 20% molar ratio of a highly purified hog kidney leucine aminopeptidase to cytochrome *c*, the liberated amino acids were separated by dialysis and the remaining partly digested cytochrome *c* isolated by column chromatography on Amberlite IRC–50. Either directly or after suitable acid hydrolysis both fractions were analysed on an automatic amino-acid analyser.

It was shown that about 30 amino acids are liberated after 20 hr of digestion from the N-terminal sequence of cytochrome *c*, and that the liberated amino acids as well as those remaining in the partly-digested protein add up rather accurately to the composition of the original cytochrome *c*. These 30 residues contain the single tryptophane, one of the two arginines, one of the three histidines, one of the three valines and one of the four prolines in the entire protein. In addition two serines, which have previously not been found in horse-heart cytochrome *c* were also liberated. Since practically the entire haem was found in the remaining partly digested cytochrome *c*, the haem must be located at least 30 amino acids away from the N-terminal end of the peptide chain.

(ii) *Amino-acid sequence of a chymotrypsin-digested cytochrome* c *'core'*. Using a partial digestion with chymotrypsin it was possible to isolate a chymotryptic 'core' of horse-heart cytochrome *c*, by column chromatography on Amberlite IRC–50 followed by high voltage paper electrophoresis. This peptide contained 42 amino acids. N-terminal and C-terminal residues were determined by reaction with fluorodinitrobenzene and carboxypeptidase digestion, respectively. The chymotryptic 'core' was digested with trypsin; the peptides formed were isolated by various combinations of paper electrophoresis and paper chromatography and their composition determined using an automatic amino-acid analyser.

A partial sequence thus established for the chymotryptic 'core' showed some unusual features. Towards the C-terminal end there was a remarkable concentration of aromatic and long-chain aliphatic amino acids, enclosing a single glutamic acid, and in the centre of the sequence a concentration of basic residues (3 or 4 lysines and one histidine). Between these two regions was located a single proline.

Mammalian heart cytochrome *c* has no known covalent bonds holding the protein together, it is readily denatured by acid and alkali and although all of its acidic and basic residues are titratable, only 8–9 of the ε-NH_2 groups of its 18 lysines will react rapidly, in the native protein, with a water soluble reagent such as 1-fluoro-2-nitro-4-benzene sulphonate. I should therefore like to propose the hypothesis that concentrations of long chain aliphatic and aromatic amino acids in specific positions provide the required hydrophobic microenvironment to stabilize particular acid-base ionic bonds which serve to keep the protein in its native folded configuration. What was observed in the chymotryptic 'core' would presumably be one such region.

Another consequence of this work is that the haem is attached at least 30 residues away from the N-terminal end and at least 30 residues away from the C-terminal end, thus putting the haem to probably within 15 residues of the centre of the peptide chain.

Comments on the Structure of Cytochrome c

PALÉUS: As to the spectrophotometric curve shown (by Margoliash) of the peptic horse haemopeptide, and the effect of addition of histidine to the solution, I want to refer to the results of Tuppy and Bodo (*Mh. Chem.* **85**, 1024, 1182, 1954), as well as those of Ehrenberg and Theorell (*Acta chem. Scand.* **9**, 1193, 1955). These authors added histidine (in the case of Tuppy and Bodo also α-benzoyl-histidine) to a solution of the tryptic horse haemopeptide of cytochrome *c* (Tuppy and Bodo) and to the peptic beef haemopeptide (Ehrenberg and Theorell) at pH 7·3 and 8·9 respectively. They found that the extinction at 550 mμ was raised to that found in native cytochrome *c*. Margoliash, however, observed this phenomenon first at pH 11.

As to the haem-linkage, I want to refer to the results of Tuppy and Bodo, who were able to show how the autoxidizable haemopeptide lost most of its autoxidizability on adding histidine at the same time as the extinction at 550 mμ of native cytochrome *c* was reached. Ehrenberg and Theorell showed spectrophotometrically the inability of ferro- and ferric protoporphyrin at pH 7 to form any compounds with lysine or glycyl-glycine.

What does Margoliash now think is the N-terminal group of his cytochrome *c* preparation? Is it histidine as he stated in 1955? Did the N-terminal group show up at the degradation of cytochrome *c* with leucine aminopeptidase, the experiment which he has shown us today? However, Minakimi, Titani and Ishikura (*J. Biochem. Tokyo*, **45**, 341, 1958) could not degrade cytochrome *c* with leucine aminopeptidase. Their results do not correspond to yours.

As to your opinion of the electrostatic holding together of the cytochrome *c* molecule it may be rather important. I also want here to mention that one can abolish the capacity of the enzyme for electron-transfer by, e.g. acetylation of the ε-NH_2-lysine group, but not by guanidation, as shown by Minakami and co-workers.

MARGOLIASH: I do not think that the results of Tuppy and Bodo (who found that adding a large excess of α-benzoylhistidine to the tryptic core of cytochrome *c* resulted in a considerable increase in the reduced α-band extinction) conflict with our own results. If I remember correctly, Tuppy and Bodo obtained an increase of the α-band extinction to about 80–90% of that of native cytochrome *c* at somewhat alkaline pH. This is very similar to our own results with imidazole and the pepsin digested cytochrome *c* 'core', and does not influence the fact that no externally added bases are required to obtain a spectrum virtually identical to that of native cytochrome *c*, at pH 11, with this 'core'. You asked whether we managed to establish an N-terminal amino acid for cytochrome *c* with leucine aminopeptidase. No, this has not been done. Leucine aminopeptidase does not produce an appreciable digestion at too low a concentration. On the other hand, at concentrations that will work, the rate of digestion is so rapid that it is not easy to establish by a kinetic study which is the first amino acid liberated. There is of course the added difficulty that if the first peptide bond to be split is broken slowly as compared to the next few bonds along the chain, all these amino acids will be liberated practically simultaneously, making it impossible to decide which is the N-terminal.

WILLIAMS: I believe that the observations of Margoliash can also be interpreted in terms of Theorell's original suggestion that the cytochrome was a di-imidazole complex. Rather than go into detail here it is proposed to submit a full analysis of these experiments elsewhere (see also, this volume, p. 141).

Structure and General Properties of Cytochrome c

Comparative Properties of Cytochrome *c* from Yeast and Heart Muscle

By J. McD. ARMSTRONG, J. H. COATES AND R. K. MORTON (Adelaide)

MORTON: This paper relates particularly to the contribution by Henderson and Rawlinson (this volume, p. 369) and describes studies briefly reported elsewhere (see Armstrong, Coates and Morton, 1958; Morton, 1958).

Air-dried baker's yeast (as used for isolation of crystalline cytochrome b_2; see Appleby and Morton, 1954, 1959) was extracted with M NaCl for about 6 hr at room temperature (about 22°C) or for 30 min at 55–60°C. The supernatant obtained on centrifuging was diluted with three volumes of water and adjusted to pH 5·7. The cytochrome *c* in the extract was absorbed batchwise onto resin (IRC-50(Na$^+$)), and after washing the resin, the cytochrome *c* was eluted either with 0·8 M NaCl in 0·1 M sodium phosphate pH 7·8, or 40% saturated ammonium sulphate pH 8·5–9·0. The cytochrome *c* was purified by chromatography on a column of the same resin until the ratio of E_{549} mμ/E_{278} mμ was constant (1·05–1·12) for the eluted cytochrome.

Electrophoresis by the moving boundary method was carried out in buffers of ionic strength of 0·2, at 11 pH values from pH 4·18 to pH 10·07. At all pH values only one protein component was detected, although some asymmetry of the boundary was apparent. Only microheterogeneity could be detected by reversal of polarity after prolonged electrophoresis at pH 9·93, which is close to the iso-electric point of the cytochrome.

Figure 1 shows the pH-mobility plot for the yeast cytochrome *c* in comparison with the plot obtained by Theorell and Åkeson (1941) and by Tint and Reiss (1950) for ox-heart cytochrome *c*. The iso-electric point for yeast cytochrome *c* is pH 9·85 ± 0·05 under our conditions, as compared with pH 10·6 for the ox-heart cytochrome *c*.

Sedimentation at 60,000 rev/min in a Spinco analytical ultracentrifuge showed only a single component. Preliminary studies using the Archibald approach-to-equilibrium procedure indicate a weight/mole of approximately 15,000 g, in reasonable agreement with the minimum weight/mole of 14,700 g estimated from the iron content of 0·38%.

The E_0' value at pH 6·4 was determined as +0·282 V by Henderson and Rawlinson (see this volume, p. 371), as compared with +0·255 V for ox-heart cytochrome *c* under similar conditions.

Table 1 is a summary of the comparative properties of yeast and heart-muscle cytochrome *c* (see also Morton, 1958). It is apparent that the two proteins are not identical. The lower iso-electric point for yeast cytochrome *c* is consistent with the lower lysine content (15–16 and 18 residues/mole in yeast and heart-muscle cytochrome *c* respectively) reported by Nunnikhoven (1958).

It is noteworthy that only one protein component was detected in these studies. Nunnikhoven (1958) extracted cytochrome *c* from yeast by Keilin's (1930) procedure, concentrated the extract on resin, and fractionated with ammonium sulphate after acidification with trichloroacetic acid (TCA). By electrophoresis at pH 8·3, two haemoprotein components were detected (containing 0·36 and 0·42% of iron for the faster and slower component respectively). Tsou (1956) obtained yeast cytochrome *c* by autolysis of dried yeast in M NaCl, concentrated it on a synthetic zeolite, eluted with 72% saturated ammonium sulphate, and precipitated the cytochrome with TCA. Two haemoprotein components were detected, capable of separation by electrophoresis at pH 6·3. The major component had an iron content of 0·43%.

However, Minakami (1955, 1956), who also avoided use of TCA, obtained only a single electrophoretic component in his preparation of yeast cytochrome *c*, which had an iron content of 0·37%, in close agreement with the results obtained in our work.

In addition to the differences indicated in Table 1, we have observed that solutions of yeast cytochrome *c* are rather unstable as compared with heart-muscle cytochrome *c*. Prolonged dialysis against distilled water at 0–2°C may lead to autoxidation of yeast ferrocytochrome *c*, and the oxidized form is no longer reduced by cytochrome b_2 and lactate. Similar results have been obtained with solutions of yeast ferrocytochrome *c* in the presence of ammonium sulphate. Denaturation of the cytochrome *c* may occur on oxidation of the reduced form with excess ferricyanide, and also on precipitation from solutions of low ionic strength with acetone at −10°C.

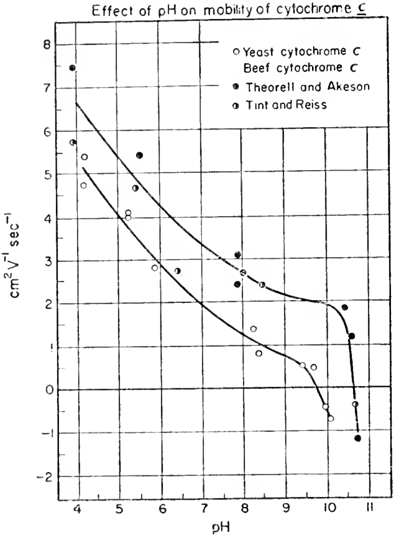

FIG. 1. pH/mobility plot for yeast ferricytochrome *c* and for heart-muscle ferricytochrome *c* (results from Theorell and Åkeson, 1941).

The prosthetic group was completely split from native yeast ferrocytochrome *c* very rapidly by treatment with silver sulphate and 2N acetic acid at room temperature (about 22°C), whereas the reaction with heart-muscle cytochrome *c* is slow under these conditions, and complete splitting requires higher temperature conditions as described by Paul (1951). However, the product obtained from the yeast cytochrome *c* appeared to be similar to those obtained by Paul from heart-muscle cytochrome *c*.

As shown in Table 1, the absorption bands of yeast ferrocytochrome *c* at room temperature are displaced slightly towards the blue as compared with the heart-muscle protein, and these differences are even more pronounced at −190°C (Estabrook, 1956). It is of interest that there is considerable similarity between the absorption spectra at −190°C of yeast ferrocytochrome *c* and of the TCA-modified heart-muscle ferrocytochrome *c* (Estabrook, 1956; and this publication, p. 444). Yeast ferricytochrome *c* obtained by Nozaki and co-workers (1957, 1958) was more readily digested by bacterial proteinase than heart-muscle ferricytochrome *c*. If the haemoprotein used

TABLE 1. COMPARATIVE PROPERTIES OF CYTOCHROME *c* OF YEAST AND OF HEART-MUSCLE

(References are given below each value or set of values)

		Yeast	Heart-muscle
Absorption bands (in mμ)	At room* temperature	α, 549·3 β, 520·0 γ, 414·7 δ, 314·0 u.v., 275 (This paper)	α, 549·8 β, 520·0 γ, 415·0 δ, 315·0 u.v., 274 (This paper)
	At −190°C	α, 546·6, 536·6 β, 526·2, 518·5, 512·3, 510·4, 507·0 (Estabrook, 1956)	α, 548·6, 545·9, 537·7 β, 528·5, 525·4, 518·5, 514·7, 511·6, 507·8, 503·1 (Estabrook, 1956)

	Yeast	Heart-muscle
E_0' (pH 6·4)	+0·282 V Henderson & Rawlinson, this volume, p. 371)	+0·255 V (Henderson & Rawlinson, this volume, p. 377)
Iso-electric pt.	pH 9·85 ± 0·05 (This paper)	pH 10·65 (Theorell & Åkeson, 1941)
Fe content (% dry wt.)	0·38% (This paper)	0·43% (Theorell & Åkeson, 1941)
Minimum wt/mole (g)	14,700 (This paper)	13,000 (Theorell & Åkeson, 1941)
$S_{20,w} \times 10^{13}$	1·7–1·9 (This paper)	1·83–1·89 (Paul 1951; Loftfield & Bonnichsen)
$D_{20,w} \times 10^{7}$	—	11·4
Partial specific volume	—	0·728 (Ehrenberg, 1957)
Molecular weight	15,000 (This paper)	13,300 (Ehrenberg, 1957; *S* and *D* data)

Amino Acid Composition	Yeast		Heart-muscle
	A	*B*	
Aspartic acid	10·9	11·8	7
Glutamic acid	8·8	9·2	11
Proline	4·2	4·8	3
Glycine	11·6	11·9	11
Alanine	7·3	8·1	5
Valine	3·0	3·3	2
Iso-Leucine	3·8	4·8	5
Leucine	6·9	5·5	5
Serine	4·3	5·8	0
Threonine	8·1	8·9	9
Cysteine	(2)	(2)	2
Methionine	1·8	2·5	2
Tyrosine	4·0	4·7	4
Phenylalanine	3·4	3·5	3
Tryptophan	(1)	(1)	1
Histidine	3·3	2·9	3
Lysine	14·8	16·0	18
Arginine	2·7	2·8	2
Ammonia	11·1	13·0	7
	Nunnikhoven (1958)		Leaf, Gilles & Pirie (1958)

* Measured at a half-band width of 1·5 Å (u.-v.) and 0·9 Å (visible).

in these studies was undenatured, the results suggest that native yeast cytochrome *c* has a more 'open' folding of the peptide chains than has native heart-muscle cytochrome *c*.

The major reason for this study was to provide well-characterized, native cytochrome *c* from yeast for use with crystalline yeast lactic dehydrogenase (cytochrome b_2). Both the yeast and heart-muscle cytochrome *c* were found to be equally reactive with the crystalline yeast enzyme. Both yeast and heart-muscle cytochrome *c* also react with heart-muscle cytochrome *c* oxidase preparations, as also found by others (Li and Tsou, 1956; Minakami, 1955, 1956).

The results thus show that both yeast and heart-muscle cytochrome *c* are closely similar enzymically but have differences in physical and chemical properties which are rather greater than the differences as yet found for cytochrome *c* preparations from different animal sources. Although the prosthetic groups of yeast and heart-muscle cytochrome *c* would appear to be similar, there are undoubtedly considerable differences in the secondary protein structure of the two cytochromes. It would appear that the reactivity of the prosthetic group of cytochrome *c* with other compounds (including enzymes such as cytochrome b_2) is relatively independent of the folding of the peptide chains of the protein.

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Properties of Native Cytochrome c

HORIO: I would like to make a comment on the properties of cytochrome *c*.

As found by Margoliash, native cytochrome *c* can be separated easily from modified cytochrome *c* by resin chromatography. Of the modified cytochrome *c* fractions, some fractions show the same absorption spectra as native cytochrome *c*.

One can recognize easily which cytochrome *c* is native, by comparing various extraction- and purification-procedures, by comparison of the properties (ascorbate-oxidizing activity, redox-potential, etc.) and by comparing the properties of each sample after treatments such as lowering and raising pH, or heating.

In the oxidized state, native cytochrome *c* is rapidly digested by a bacterial proteolytic enzyme. The modified cytochrome *c* is much more rapidly digested. This fact ndicates that the modified cytochrome *c* has a more unfolded protein-structure than native cytochrome *c*. With the same enzyme, native cytochrome *c* crystallized from baker's yeast is much more rapidly digested than those from ox- and horse-heart.

Using native cytochrome *c*, the reduced form is much more resistant to digestion by the proteolytic enzyme than the oxidized form. In fact, if the rapid autoxidation of the reduced cytochrome *c*, (which is catalyzed by proteolytic products obtained by digestion of oxidized cytochrome *c*) is prevented by using anaerobic conditions, the reduced form is not attacked by the proteolytic enzyme at all.

On the other hand, if one uses crystalline enzymes, for example, bacterial amylase, yeast alcohol dehydrogenase, animal triose-phosphate dehydrogenase, animal lactate dehydrogenase, the enzymes in their enzymically-active state are not digested by the proteolytic enzyme, while the denatured enzymes (regardless of whether the denaturation is reversible or irreversible), are rapidly digested. Kunitz and co-workers have reported that crystalline yeast hexokinase is digested by trypsin and that the enzyme becomes more resistant against the digestion in the presence of the substrates than in the absence (glucose > fructose > mannose).

These considerations indicate that the reduced form of cytochrome *c* has a more rigidly folded protein-configuration than the oxidized form.

As mentioned by Smith in connexion with estimation of cytochrome *c* oxidase activity (p. 260), using cytochrome *c* modified in its monomer state, one may not find serious differences in enzymic reduction and oxidation of native and modified cytochrome *c*. But generally speaking, enzyme proteins lose their enzymic activity at the same time as they lose the folded (coiled) protein-configuration. This concept does not fit with the enzymic reduction and oxidation of modified cytochrome *c*. From this point of view, I am just wondering what function is coupled with the folded configuration of the native cytochrome *c*, and with the alteration in the folded protein-configuration which may result from the oxidation-reduction reaction of cytochrome *c*.

Reactivity of Native Cytochrome c *in Oxidative Phosphorylation*

MORRISON: As Henderson and Rawlinson (p. 369) have pointed out, the modified heart-muscle cytochrome *c* (fraction 2) obtained by the chromatographic method of Margoliash does not appear to have markedly decreased biological activity as judged by its ability to transport electrons. This is not true when the role of cytochrome *c* in oxidative phosphorylation is assayed (Morrison, Hollocher and Stotz, *Arch. Biochem. Biophys*. **92**, 338, 1961.

When this modified cytochrome *c* fraction was added to tightly-coupled rat liver mitochrondria, there was no increase in this rate of phosphorylation with added cytochrome *c*. This is in marked contrast with the results obtained with the so-called 'native' fraction of cytochrome *c* (fraction 1).

These results point to the direct participation of the cytochrome *c* molecule in the oxidative phosphorylative mechanism.

Structure of Bacterial Cytochromes of c*-type*

POSTGATE: My question is addressed to Margoliash. Several of the bacterial cytochromes of the *c*-type are neutral or acid (see Morton, *Rev. pure appl. Chem*. **8**, 161, 1958). If electrostatic links between polar groups in the protein are responsible for the very great thermal stability of this group of proteins, would not this thermostability be pH-dependent? If so, would not a study of the pH-dependence of the thermostability of a basic and a neutral cytochrome *c* show differences bearing on the nature of the linkage in folding of the peptide chain?

MARGOLIASH: My idea of hydrophobically shielded electrostatic bonds as the bonds conferring on cytochrome *c* its native folded configuration and relative resistance to some types of denaturation procedures, such as heating, was advanced for the common mammalian types of cytochrome *c* which are strongly basic proteins. The thermostability of such cytochromes *c* is certainly pH-dependent.

There is, however, no particular reason why similar bonds could not occur in neutral or acid cytochromes *c*, since all that is required is a concentration of hydrophobic amino-acid side chains around the region where such an electrostatic bond may be formed. The effect of such an 'umbrella' would be to change the dielectric constant at the point where the electrostatic bond is to be formed, so as to render it more stable than it would be in a normal water environment. Without the specific hydrophobic microenvironment an electrostatic bond would be quite unstable even at neutral pH in an aqueous medium.

Structure and Redox Potentials of Cytochrome c

The effect of denaturation on E_0' values for cytochrome c

MARGOLIASH: To obtain what we have called the trichloroacetic acid modified or denatured cytochrome *c* it is necessary to have the cytochrome *c* in solution during the denaturation procedure, and not precipitated. This is done by carefully controlling the pH. Under such conditions, or by using ethanol as a denaturing agent, one obtains a product that can be separated into fractions of increasing degree of denaturation by column chromatography on Amberlite IRC-50. These fractions have the full complement of amino acids present in the original native protein. The more the degree of denaturation, the lower the E_0' value. The actual values obtained range from that of native cytochrome *c* at +0·255 V to not far from 0·0 V.

The ligands to iron in cytochromes of the c*-type*

PERRIN: The difference of 0·54 V in E_0' between cytochrome *c* and cytochrome *c* peptide indicates that in the latter the ferric form is stabilized by a factor of 10^9 relative to the ferrous form, compared with cytochrome *c*. This represents a preferential stabilization of the ferric cytochrome *c* peptide by a ΔG of about 13 kcal. This difference is much greater than is found in the iron porphyrin haemochromes where the ligands are neutral molecules and I should like to suggest that in the peptide the sixth link to the metal is through a carboxyl group (e.g. of glutamic acid) or some other anion because anions form much more stable complexes with ferric than with ferrous iron. The very large difference between cytochromes c_2 and c_3 would then indicate that in the former the sixth position ligand is neutral (e.g. amino-N or imidazole-N) while in the latter it is carboxyl. Falk and I (p. 66) have indicated why we think that the absorption spectra of such complexes are insensitive to the particular ligands other than the porphyrin groups.

MARGOLIASH: I wish to reply to Perrin's suggestion that the lowering of the E_0' value of cytochrome *c* on denaturation may be due to an exchange of a —COO⁻ group for one of the normal haemochrome-forming ligands in the native protein, during denaturation as well as during proteolytic digestion to form the 'core'.

I think that the spectrophotometric evidence rules out such a possibility. Whether a ligand is acidic or basic, the spectrophotometrically-observed p*K* for the dissociation of a particular ligand would be at a pH below that of the normal p*K* value for the ligand in solution, since as pointed out previously by Perrin, one must take into consideration the constants involved in the reaction of the ligand with the haem iron. In the ferro- form of the cytochrome *c* 'core', the lowest apparent p*K* value we have observed spectrophotometrically, using the visible region of the spectrum, was at pH values between 5 and 6. This clearly rules out the participation of a carboxyl as a haemochrome-forming group in the reduced form of the 'core'.

With reduced denatured cytochrome *c* the results will naturally vary with the degree of denaturation of the fraction studied, all the way from an apparent p*K* at p*K* 2–3 for the native protein, to a behaviour essentially identical to the 'core' for the most denatured material, again showing the lowest p*K* at pH values of 5–6. Since as discussed previously, the E_0' value will decrease with an increasing degree of denaturation and one would expect that the introduction of a carboxyl group would result in a decrease in E_0', Perrin's suggestion cannot be reconciled with the spectrophotometric results, at least for those derived forms of the protein we have studied, which show pK values apparently shifting towards the alkaline side as denaturation proceeds.

I would, however, like to stress that our results with the E_0' values for denatured cytochrome *c* fractions are compatible with the theory that it is not the particular ligands involved that establish the properties peculiar to the cytochrome *c* haemochrome as contrasted with those of an ordinary chemical haemochrome, but rather the native configuration of the protein. This holds not only for the haemochrome properties I discussed following Paléus' paper, but also for the oxidation-reduction potentials.

Influence of dithionite on redox potential of cytochrome c

HENDERSON: According to Paul (*The Enzymes*, Academic Press Inc., New York, 1951) dithionite when used to titrate electrometrically cytochrome *c* gives the low value of +0·12 V instead of +0·26 V obtained with other reductants. This may be the explanation for the similar value of Green (*Proc. roy. Soc.* **B114**, 423, 1934) for yeast cytochrome c.

This would presumably be due to some compound formation with the cytochrome *c*. Has Estabrook observed any difference in the low-temperature spectra between cytochrome *c* reduced with dithionite and the same material reduced by other means, as they might show such compound formation?

ESTABROOK: We have used mainly enzymic reduction where possible to try and detect any difference in spectra and find that there is no obvious difference between the spectral properties at low temperature of samples of purified haemoproteins reduced enzymically or by dithionite treatment. In the cases of the haemopeptide of cytochrome *c* or the alkaline haemochromes there was, of course, only dithionite reduction employed.

THE ELECTRON TRANSFER FROM CYTOCHROMES TO TERMINAL ELECTRON ACCEPTORS IN NITRATE RESPIRATION AND SULPHATE RESPIRATION

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Until 1949 the cytochrome system was considered to participate only in the electron transfer to oxygen. The participation of a cytochrome in anaerobic oxido-reduction was first suggested by Sato and Egami (1949) in the case of nitrate reduction in *E. coli*. Recently a cytochrome was found even in a strict anaerobe, Postgate (1954) and Ishimoto, Koyama and Nagai (1954a, b) independently demonstrating the participation of a cytochrome in the reduction of sulphur compounds in *Desulphovibrio desulphuricans*.

Nitrate reduction with participation of a cytochrome system has been called by us 'nitrate respiration', because its physiological meaning is not nitrogen assimilation, but energy-yielding through oxido-reduction similar to aerobic respiration (Sato, 1950; Taniguchi, Sato and Egami, 1956). The electron transport mechanism functioning in such processes also appears to resemble that of aerobic respiration. It has been shown that the electron-transferring system in nitrate respiration in *E. coli* resides on particulate structures containing cytochrome b_1 as in the case of the analogous system involved in aerobic respiration. On the other hand, enzyme systems of different nature in soluble form without participation of cytochromes have been shown to be responsible for the reduction of nitrate in 'nitrate assimilation' (Nason, 1956).

Similarly sulphate reduction in *Desulphovibrio desulphuricans*, being an energy-yielding mechanism with participation of cytochrome c_3, may be called 'sulphate respiration'.

Our studies on the mechanisms of enzymic electron transfer in nitrate respiration and sulphate respiration until 1957 were summarized by Taniguchi, Asano, Iida, Kono, Ohmachi and Egami (1958) and Ishimoto, Kondo, Kamayama, Yagi and Shiraki (1958) respectively at the International Symposium on Enzyme Chemistry held in Tokyo in 1957. However, the electron transfer from the cytochromes to corresponding terminal electron acceptors had been scarcely investigated on the enzyme level till the symposium.

The present paper describes the recent results obtained in Egami's laboratories by Taniguchi, Ohmachi and Itagaki and by Ishimoto and Fujimoto on the terminal electron transfer in nitrate respiration and sulphate respiration respectively.

NITRATE RESPIRATION

Cytochrome b$_1$ *and Nitrate Respiration*

A highly active nitrate-reducing system of nitrate respiration type was found in *E. coli* grown in anaerobiosis with nitrate. As shown in Table 1, anaerobic cells had very little activity in the aerobic reduction of nitrate. A remarkably opposed tendency could be seen in cells grown aerobically in the same medium with nitrate. They exhibited aerobic reduction much more than anaerobic reduction (Table 1).*

TABLE 1. NITRATE-REDUCING ACTIVITY NaR OF *E. coli* CELLS*

Reaction mixture contains 100 μmoles of KNO_3, 1 μmole of formate, 240 μmoles of phosphate buffer, pH 7·1, and *E. coli* cells in total volume of 3·0 ml. Temperature: 30°C.

Conditions of nitrate reduction *in vitro*	Nitrate-reducing activity of cells under various growth conditions (in μmoles of NO_3^- reduced/mg N/hr)	
	Aerobic	Anaerobic
Aerobic† ($\rightarrow NH_3 \rightarrow \ldots$)	30–50	0–5
Anaerobic‡ ($\rightarrow NO_2^-$)	10–20	200–500

† In Warburg vessels under vigorous shaking.
‡ In Thunberg tubes under anaerobic conditions.

The addition of nitrate in the anaerobic culture stimulates not only the bacterial growth but also the cytochrome b_1 content of the cells to the extent of 2 to 3 times higher (almost to the same level as that of aerobic cells) as compared with that of cells grown in anaerobiosis without nitrate.

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* *Abbreviations*

The following abbreviations have been used in this paper: nitrate reductase, NaR; cytochrome, cyt.; formate dehydrogenase, FDH; *p*-chloromercuribenzoate, PCMB; reduced methyl- or benzylviologen, MVH or BVH; phenazinemethosulphate, PMS; methylene blue, Mb; flavin adenine dinucleotide, FAD; flavin mononucleotide, FMN; adenosine-5′-phosphosulphate, APS; 3′-phosphoadenosine-5′-phosphosulphate, PAPS; adenosine triphosphate, ATP; 2-heptyl-4-hydroxyquinoline-N-oxide, HOQNO; diphosphopyridine nucleotide, DPN; reduced di- or tri-phosphopyridine nucleotide, DPNH or TPNH; 2-amino-2-hydroxymethylpropane-1:3-diol, Tris.

The anaerobic cells cultured anaerobically with nitrate and containing the active mechanism of nitrate respiration were used throughout this study.

The nitrate reductase activity was found in the particulate fraction (sedimented at 2,000–20,000 *g*) with cytochrome b_1 and formate dehydrogenase after alumina or sonic destruction of cells (Table 2). This particulate

TABLE 2. DISTRIBUTION OF NITRATE REDUCTASE, FORMATE DEHYDROGENASE AND PROTOHAEM IN CELLULAR FRACTIONS OF *E. coli*

4·0 g of wet cell paste was ground with alumina and subjected to the following centrifugal fractionation. All determinations at 30°C.

Fraction	Total N (mg)	NaR activity*		FDH activity†		Protohaem	
		Total	Specific	Total	Specific	Total	mμg/mgN
Original (2,000 *g* extracts)	36·5	4250	117	1880	51	1860	51
Large particle (2,000–20,000 *g*)	10·2	2360	235	3400	340	1230	121
Small particle (20,000–110,000 *g*)	2·9	224	77	50	160	300	103
Soluble fraction (110,000 *g*)	26·5	235	9	0	0	450	17

* The donor for NaR was MVH. One unit of NaR is defined as an amount of the enzyme which produces 1 μmole of nitrite in 1 hr. Specific activity is defined as units/mg N.

† Acceptor for FDH was Mb. Activity is expressed as the amount of enzyme which reduces 1 μmole of Mb in 1 hr.

TABLE 3. COMPARISON OF ELECTRON DONORS FOR PARTICULATE NITRATE REDUCTASE ACTIVITY

Electron donors (reduced compound in each case‡)	NaR activity (relative value)
Methylviologen	100
Benzylviologen	100
Phenosafranine	58
Pyocyanine	55
Methylene blue	5·3
Phenazine methosulphate	2·7
FAD	29
FMN	31
Formate	16
DPN	6·8

‡ Electron donors except formate and DPNH were reduced by dithionite. All compounds at 2×10^{-3} M concentration except reduced FAD and reduced FMN (both at 2×10^{-4} M) and formate (5×10^{-2} M).

fraction was found to be devoid of not only dehydrogenase activity (using methylene blue (Mb) for assay) for lactate, succinate, β-hydroxybutyrate and glucose, but also hydrogenase (production of H_2 from reduced methylviologen, MVH), and of reduced tri- and di-phosphopyridine nucleotide (TPNH and DPNH) oxidase activity. Contrary to the very high activity of formate dehydrogenase and oxidation of formate by nitrate, aerobic oxidation of formate was found to be weak. Thus, the particle seems to be highly characterized by the well-developed formate dehydrogenase—cytochrome b_1—nitrate reductase system, i.e. the enzyme system of nitrate respiration type. Nitrate reduction by the particulate nitrate reductase with various electron donors is summarized in Table 3.

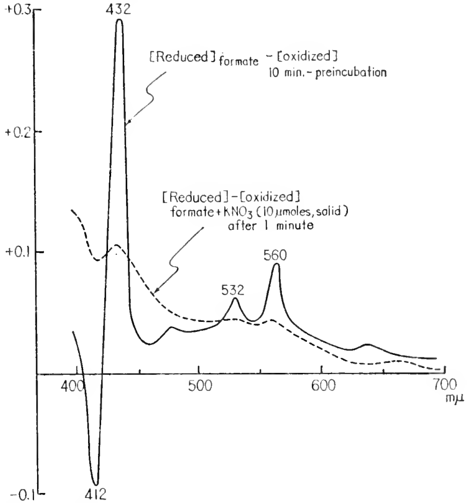

Fig. 1. Difference spectra of particulate preparation. The solution in Thunberg tube-type cuvette contained large particles (0·6 mg N/ml), phosphate buffer 50 μmoles, pH 7·1 and formate 50 μmoles in 3·0 ml under anaerobic conditions at 30°C.

Cytochrome b_1 in the particulate fraction was rapidly reduced with DPNH or even more rapidly with formate under anaerobic conditions. TPNH was quite inactive as an electron donor. Cytochrome b_1 thus reduced was rapidly reoxidized with nitrate (Fig. 1).

In this case nitrate reduction was strongly inhibited by 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), a specific inhibitor for cytochrome b_1. On the contrary, nitrate reduction by reduced dyes such as MVH was not inhibited by HOQNO (Table 4).

TABLE 4. INHIBITION AND ACTIVATION OF THE PARTICULATE NITRATE REDUCTASE ACTIVITY BY SEVERAL REAGENTS

Reagent	Concentration (M)	Inhibition (%)	
		With formate	With MVH
Amytal	2×10^{-3}	75	-20*
Menadione	5×10^{-4}	-40*	0
Dicoumarol	3×10^{-5}	70	25
Dicoumarol + menadione	3×10^{-5} 5×10^{-4}	70	—
HOQNO	10^{-5}	85	0
PCMB	5×10^{-4}	60	20
PMS	$3{\cdot}3 \times 10^{-4}$	-160*	5

* Activation.

Nitrate reduction by the particulate system with formate as an electron donor was strongly inhibited not only by CN^- and N_3^-, but also by dicoumarol, amytal and *p*-chloromercuribenzoate (PCMB). This finding suggests the participation of flavoprotein, dicoumarol-sensitive factor and SH group besides heavy metals in the electron transfer sequence. On the other hand it was activated by vitamin K_3 and phenazine methosulphate (PMS) (Table 4).

From these findings the following electron transfer sequence for the particulate system can be proposed:

$$\begin{tikzcd}
\left.\begin{array}{l}\text{Formate}\\ \text{DPNH}\end{array}\right\} \arrow[r] & \text{(FAD)} \arrow[r] & \text{Cyt. } b_1 \arrow[r] & \text{Nitrate Reductase (NaR)} \arrow[r] & NO_3^- \\
 & & & \text{MVH} \arrow[u] &
\end{tikzcd}$$

Phosphorylation Coupled with the Electron Transfer

The decrease of inorganic phosphate was observed coupled with nitrate reduction with formate as an electron donor by the particulate fraction prepared with 2-amino-2-hydroxymethylpropane-1:3-diol (Tris) buffer (0·02 M, pH 7·2). Adenosine triphosphate (ATP), glucose, yeast hexokinase, Mg^{++} and F^- were added in the reaction mixture besides inorganic phosphate. Glucose was scarcely utilized as an electron donor. It was found that the particulate fraction itself contained ATP-ase which was activated by Mg^{++} and inhibited by F^-.

The decrease of inorganic phosphate during the nitrate reduction indicates the occurrence of an anaerobic phosphorylation coupled with the electron transfer from formate to nitrate through cytochrome b_1. These experiments are still in a preliminary stage and the fate of phosphate incorporated remains to be elucidated.

Solubilization and Purification of Particulate Nitrate Reductase

The greater part of nitrate reductase, estimated with MVH as an electron donor, of the particulate fraction was solubilized, when heat-treated at 60°C for 5 min and kept at pH 8·3, at 4°C for 20 hr. Formate dehydrogenase and cytochrome b_1 were not solubilized by the procedure. Nitrate reductase thus solubilized was purified to about 1,000 times the activity of cell paste (yield 10–20%) by adsorption onto and elution from calcium phosphate gel, ammonium sulphate fractionation, and ultracentrifugation (110,000 *g*, 3–4 hr). The purified nitrate reductase with yellowish-brown colour is homogeneous in ultracentrifugal and electrophoretic analysis and has a specific activity as high as about 200,000 μmoles NO_2^- formed/mgN/hr (Table 5).

TABLE 5. SOLUBILIZATION AND PURIFICATION OF NITRATE REDUCTASE

Step	Fraction	N (mg)	NaR activity (units × 10^{-4})	Specific activity (units/mg N × 10^{-3})
1	Frozen cell paste	270 g wet wt.		0·18
2	Large particle (2,000–20,000 *g*)	2500	135	0·54
3	Supernatant after heat treatment and incubation in cold	580	100	1·72
4	Calcium phosphate gel eluate	227	90	3·95
5	$(NH_4)_2SO_4$—1st ppt. (38–53% saturation)	55·2	80	14·5
6	$(NH_4)_2SO_4$—2nd ppt. (30–40% saturation)	11·2	50	56·2
7	1st pellet of ultracentrifuge (110,000*g*, 3–4 hr)	3·04	40	132
8	3rd pellet of ultracentrifuge	1·50	28	186

It has no specific absorption peak except that of protein (275–280 mμ) and the absorbance decreases gradually over the entire near-ultra-violet and visible region with increasing wavelength. The difference spectrum, (oxidized − reduced enzyme) showed a broad peak around 445–450 mμ which instantly disappeared on the addition of nitrate, with simultaneous production of nitrite (Fig. 2).

The purified enzyme preparation contains about 40 atoms of Fe per molecule, on the basis of its weight of approx. 10^6 g/mole, as estimated from measurement of sedimentation constant, $S^0_{20,\,w} = 25{\cdot}0\ s$, and diffusion coefficient, $D_{20,\,w} = 2{\cdot}27 \times 10^{-7}\ \mathrm{cm^2\,sec^{-1}}$, and assuming its partial specific volume (V_{20}) as 0·75 ml/g. By emission spectrographic analysis using the cathode layer method, the existence of Mo (1 atom/molecule) besides Fe was observed (Table 6). Analysis of flavin in the homogeneous preparation, as recorded in Table 7, led to the conclusion that the flavin content in the enzyme is close to the limit of error of determination. So the flavin content is, if any, only a trace and the enzyme may be regarded as a metalloprotein but not as a metalloflavoprotein. In our previous preliminary communication (Taniguchi and Itagaki, 1959), it was considered to be a metalloflavoprotein.

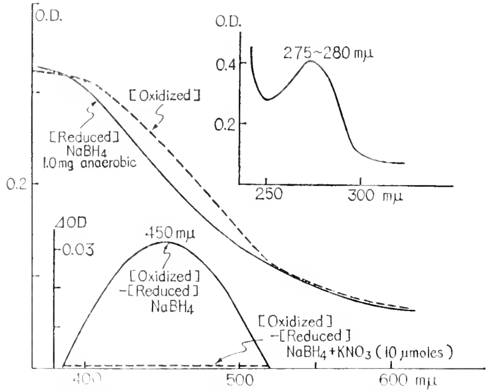

FIG. 2. Absorption spectrum of purified nitrate reductase. The solution contained NaR preparation (specific activity: 15 × 10⁴ units/mg N) in final concentration of 1·9 mg protein/ml in phosphate buffer 0·05 M, pH 7·1 in Thunberg tube-type cuvette at 30°C.

Purified solubilized nitrate reductase does not reduce nitrate with formate or DPNH, but reduces nitrate with MVH and, less actively, with reduced flavin adenine dinucleotide (FAD), riboflavin phosphate (FMN) and free riboflavin (Table 8). CN^- and N_3^- strongly inhibit the reaction (Table 9). CO (1 atm in the dark) has no effect.

Nitrate reductase thus obtained may be regarded as a solubilized form of the terminal oxidoreductase in the particulate fraction and so is an anaerobic variant of terminal oxidases (Fig. 3).

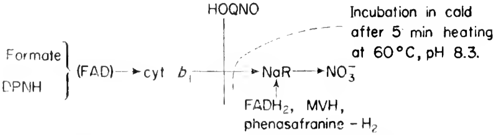

FIG. 3. Particulate nitrate-reducing system.

TABLE 6. METAL CONTENT OF PURIFIED NITRATE REDUCTASE

Experiment	Specific activity (units/mg N $\times 10^{-4}$)	Duration of dialysis in hours*	Number of atoms/NaR molecule on the basis of its molecular weight (10^6)	
			Mo	Fe
1	16	20	1·2–0·8†	45
2	18	15	—	43
		50	1·1–0·7‡	36

* Dialysis was against deionized water.
† Internal standard method with Pd and V.
‡ Visual method.

TABLE 7. CONTENT OF FLAVIN IN NITRATE REDUCTASE

Experiment	Specific activity of NaR (units/mg N $\times 10^{-4}$)	Content of riboflavin in μg/mg dry wt	Number of flavin molecules/NaR molecule
1	22	2·8	0·009
2*	22*	0·0	0·000
3	18	0·8	0·002
4	15	0·2	0·0005
5	20	—	0·02†

* The sample used was the same preparation as in Experiment 1 but with preliminary digestion by crystalline chymotrypsin (8% of weight of NaR) for 3 hr at pH 8·15, 37°C.

† This value was obtained by D-amino acid oxidase assay.

TABLE 8. COMPARISON OF ELECTRON DONORS FOR PURIFIED NITRATE REDUCTASE ACTIVITY

Electron donors (reduced compound in each case)	V_{max} (relative value)	K_m for the donor (M $\times 10^5$)
Methylviologen	100*	<0·1†
Benzylviologen	100*	<0·1†
Phenosafranine	42	<0·1
Methylene blue	1·4	4·5
FAD	0·6	1·3–2·0
FMN	3·0	2·6
Riboflavin	1·6	1·6

* For these one-electron donors, the figures should be halved for the comparison on the basis of nitrite-producing rate.

† These true figures are known to be much less than that for reduced phenosafranine.

TABLE 9. EFFECTS OF METAL-BINDING REAGENTS ON PURIFIED NITRATE REDUCTASE

Part A

	Inhibition (%) for conc. shown:			
Inhibitor	10^2 M	10^3 M	10^4 M	10^5 M
KCN	98	75	20	0
NaN_3	100	100	75	60
Nitroso-R	70			
Tiron	40			
Thiourea	45			

Part B

Inhibitor	Inhibition (%)
α, α′-dipyridyl, 8-hydroxyquinoline, *o*-phenanthroline, diethyldithiocarbamate (all at 10^{-3} M), allyl thiourea (5×10^{-4} M), *o*-nitrosoresorcinol monomethyl ether ($2{\cdot}4 \times 10^{-4}$ M)	10–30
NaF, EDTA (10^{-2} M), dithizon (8×10^{-5} M), salicylaldoxime (10^{-3} M)	0

TABLE 10. COMPARISON BETWEEN NITRATE REDUCTASE OF RESPIRATION TYPE OF *E. coli* AND NITRATE REDUCTASE OF ASSIMILATION TYPE OF *Neurospora*

	E. coli	*Neurospora*
Physiological role	Nitrate respiration	Nitrate assimilation
Cytochrome participation	Cytochrome b_1	None
Natural electron donor	Cytochrome b_1	TPNH
Preferable artificial electron donor	MVH	Reduced trichloro-indophenol
Specific activity	~200,000 (with MVH)	~80 (with TPNH)
K_m for nitrate	$8{\cdot}1 \times 10^{-4}$ M	$1{\cdot}4 \times 10^{-3}$ M
Cytochrome *c* reductase activity	None	Active
Enzyme-bound components:		
flavin	Not detectable	FAD
Fe	~40 atoms/molecule	Not reported
Mo	1 atom/molecule	Present

The properties of *E. coli* nitrate reductase of respiration type are summarized in Table 10 in comparison with nitrate reductase of assimilation type from *Neurospora*. The electron transport sequence of these two nitrate reductase systems is shown in Fig. 4.

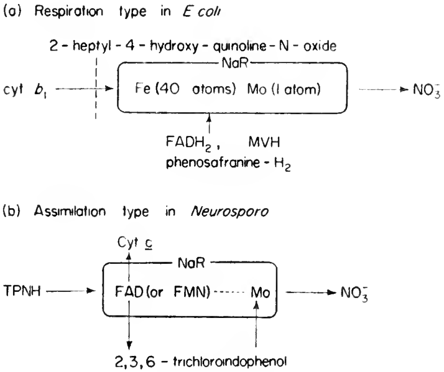
(a) Respiration type in *E coli*
2 - heptyl - 4 - hydroxy - quinoline - N - oxide
NaR
cyt b_1
Fe (40 atoms) Mo (1 atom)
NO_3^-
$FADH_2$, MVH
phenosafranine - H_2
(b) Assimilation type in *Neurospora*
Cyt c
NaR
TPNH
FAD (or FMN) Mo
NO_3^-
2,3,6 - trichloroindophenol

FIG. 4. Electron transport system leading to nitrate.

SULPHATE RESPIRATION

Pathway of Sulphate Reduction

Although extracts obtained from cells of *Desulphovibrio* by sonic or alumina destruction had ability to reduce sulphite or thiosulphate to sulphide by hydrogen, they did not reduce sulphate. Recently, Ishimoto (1959) and Peck (1959) found independently that sulphate was reduced to sulphide in the presence of ATP in the extracts. Hydrogen uptake by the extracts, fortified with methylviologen as an intermediary electron carrier, was measured with Warburg manometers in a hydrogen atmosphere and the amounts of hydrogen sulphide developed and absorbed in alkali in the centre wells were determined colorimetrically (Table 11). The ratio of amounts of the added sulphate, the adsorbed hydrogen and the formed sulphide was 1:3·5:1·02, which indicates the complete reduction of sulphate to sulphide:

$$SO_4^{--} + 4H_2 = S^{--} + 4H_2O$$

The formation of adenosine-5′-phosphosulphate (APS) from sulphate and ATP by the extracts in the absence of hydrogen was demonstrated by radioautography. For this purpose, paper electrophoresis (ammonium acetate buffer, pH 5, 3 hr, 12 V/cm) and paper chromatography (solvent: *iso*butyric acid–ammonia and propanol–ammonia) were employed (Ishimoto and Fujimoto, 1959).

TABLE 11. SULPHATE REDUCTION IN THE PRESENCE OF ATP

Reaction mixture in Warburg vessels contained 50 μmoles of Tris buffer, pH 7·4, 28 μmoles of NaF, 1 μmole of methylviologen and crude extracts of sulphate-reducing bacteria in total volume of 1·4 ml. Centre wells contained 0·2 ml of 2 N NaOH. Gas phase: hydrogen. Temperature: 30°C.

Additions (μmoles)	Hydrogen uptake (μmoles)	H_2S formed (μmoles)
(1) K_2SO_4, 10	−0·1	0·15
(2) ATP, 10	3·3	0·90
(3) K_2SO_4 2, ATP, 10	10·3	2·95
(4) K_2SO_4 10, ATP, 2	5·9	1·45
(3) minus (2)	7·0	2·05
(4) minus (1)	6·0	1·31

Synthetic APS (Baddiley, Buchanan and Letters, 1957) was reduced in similar conditions but without ATP. The ratio of hydrogen uptake and development of hydrogen sulphide was 4 : 1 (Table 12).

TABLE 12. REDUCTION OF ADENOSINE-5′-PHOSPHOSULPHATE

Reaction mixture in Warburg vessels contained 50 μmoles of Tris buffer, pH 7·0, 1 μmole of methylviologen, 0·2 ml of crude extracts and 1·64 μmoles of APS in a total volume of 0·9 ml. Centre wells contained 0·2 ml of 2 N NaOH. Gas phase: hydrogen. Temperature: 30°C. Control vessel was without APS.

	APS added (μmoles)	Hydrogen uptake (μmoles)	H_2S formed (μmoles)
Experiment	1·64	4·02	1·21
Control	0	−0·54	0·13
Difference		4·56	1·08

The disappearance of APS and formation of adenylic acid was demonstrated by paper electrophoresis and by paper chromatography. These results indicate the pathway of sulphate in the reduction as follows (Ishimoto and Fujimoto, 1959):

$$SO_4^{--} + ATP = APS + 2P_1 \text{ (or PP)} \quad \text{by sulphurylase}$$

$$APS + 2e = AMP + SO_3^{--} \quad \text{by APS reductase}$$

$$SO_3^{--} + 3H_2 = S^{--} + 3H_2O \quad \text{by sulphite reducing system.}$$

The intermediary formation of sulphite was shown by the inhibitory experiments with arsenite (0·001 M), a strong inhibitor for sulphite reductase.

The retardation of hydrogen absorption after 1 mole uptake was observed in the presence of arsenite (Fig. 5).

The formation of sulphite was demonstrated with partially-purified enzyme preparation, which had activity to reduce APS but not sulphite or sulphate in the presence of ATP (Table 13).

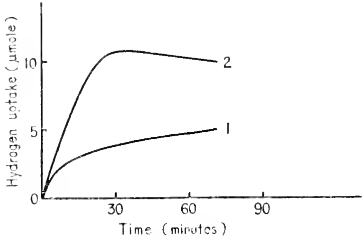

FIG. 5. Inhibition of APS reduction by arsenite.
1: with AsO_2^-, 2: without AsO_2^-.

TABLE 13. REDUCTION OF ADENOSINE-5′-PHOSPHOSULPHATE TO SULPHITE BY PARTIALLY-PURIFIED ENZYME

Reaction mixture contained 50 μmoles of phosphate buffer, pH 7·0, 1 μmole of methylviologen, 1·0 ml of partially-purified enzyme preparation (10 mg organic substance) and 2·75 μmoles of APS in total volume of 2·0 ml. Centre wells contained 0·2 ml of 2 N NaOH. Gas phase: hydrogen. Temperature: 30°C. Preparation of the enzyme was as follows. The crude extracts obtained by sonic disintegration were treated with ammonium sulphate and precipitate between 33 % and 67 % saturation was dissolved in 0·05 M-phosphate buffer, pH 7·0. After dialysis, calcium phosphate gel adsorption was carried out. This first eluate with 0·125 M phosphate buffer, pH 7·0, could reduce APS, but hardly reduce sulphite to sulphide, as shown below.

APS added (μmoles)	Hydrogen uptake (μmoles)	SO_3^{--} formed (μmoles)	H_2S formed (μmoles)
2·75	2·32	1·90	0·0

These results agree with those of Peck (1959), which showed also the intermediary formation of sulphite as well as the stoichiometry of related phosphorus metabolism.

3′-Phospho-adenosine-5′-phosphosulphate (PAPS) which is known as 'active sulphate' involved in esterification of sulphate (Robbins and Lipmann, 1957) and as an intermediate in sulphate reduction in yeast extracts (Wilson and Bandurski, 1958; Hilz and Kittler, 1958), was not reduced under similar conditions and proved to have no effect on sulphate reduction in the presence of ATP in the extracts (Table 14). The participation of PAPS was thus excluded.

TABLE 14. REDUCTION OF 3′-PHOSPHO-ADENOSINE-5′-PHOSPHATE

Reaction mixture contained 60 μmoles of phosphate buffer, pH 7·2, 50 μmoles of NaF, 1 μmole of methylviologen and 0·5 ml of crude extract in total volume of 1·8 ml. Centre wells contained 0·2 ml of 2 N NaOH. Gas phase: hydrogen. Temperature: 30°C.

Additions (μmoles)			Hydrogen uptake (μmoles)	H_2S formed (μmoles)
PAPS	ATP	K_2SO_4		
0·71	5	20	5·0	1·19
0·71	0	20	0·4	0·34
0	5	20	5·4	1·21
0	0	20	0·2	0·14

Intermediary formation of APS by the consumption of ATP in the course of sulphate reduction may be conceived to elevate the oxido-reduction potential of the system, sulphate-sulphite, above that of cytochrome c_3 (Table 15).

TABLE 15. OXIDATION-REDUCTION POTENTIAL OF ACIDS OF SULPHUR

	Reaction	E_0' (V)
1	$SO_4^{--} + H_2 = SO_3^{--} + H_2O$	−0·486
2	$SO_3^{--} + 3H_2 = S^{--} + 3H_2O$	−0·120
3	$S_2O_3^{--} + H_2 = H_2S + SO_3^{--}$	−0·423
4	$S + H_2 = H_2S$	−0·276
5	$APS + H_2 = AMP + SO_3^{--}$	0·0

Effect of Cytochrome c$_3$ *on Sulphate Reduction*

In the reduction of sulphate in the presence of ATP as well as in the reduction of APS, omission of methylviologen from the reaction mixture retarded the reaction, especially when extracts deprived of cytochrome c_3 by passage through a column of cation-exchanger (Amberlite IRC50) were employed as enzyme solutions. The addition of cytochrome c_3 to the solution stimulated the hydrogen uptake in the case of sulphate reduction in the presence of ATP as well as in the case of APS reduction. Similar results were obtained with partially-purified enzyme, which reduced APS to sulphite, in the presence of hydrogenase. Mammalian cytochrome *c* was quite ineffective. These results (Table 16) indicate the indispensable roles of the cytochrome c_3 as an electron carrier in the reduction of APS to sulphite as well as in the reduction of sulphite to thiosulphate (Ishimoto *et al.*, 1957, 1958).

TABLE 16. EFFECT OF CYTOCHROME c_3 ON THE REACTION RATE OF SULPHATE AND APS REDUCTION

(*a*) The extracts obtained by sonic disintegration were centrifuged at 100,000 *g* for 1 hr and the supernatant was passed through the column of Amberlite-IRC50 (NH_4^+ type) to eliminate cytochrome c_3. The solution obtained was used as an enzyme preparation. Warburg manometers were employed and hydrogen uptake was measured in a hydrogen atmosphere. Temperature: 30°C. The reaction mixture contained the enzyme preparation (1·61 mg N), hydrogenase preparation (0·70 mg N) (Ishimoto *et al.*, 1957), 50 μmoles of Tris buffer, pH 7·2, 20 μmoles of NaF, 10 μmoles of K_2SO_4 and 5 μmoles of ATP. Total volume was 1·3 ml. (*b*) APS reduction to sulphide. The conditions were similar to (*a*) but reaction mixture contained the enzyme preparation (1·07 mg N), hydrogenase preparation (0·34 mg N), 50 μmoles of Tris buffer, pH 7·2, and 2·1 μmoles of APS in total volume of 0·8 ml. (*c*) APS reduction to SO_3^{--}. The reaction mixture contained the partially-purified enzyme (the same sample in Table 13) (0·35 mg organic substance), hydrogenase preparation, 50 μmoles of phosphate buffer, pH 7·0, and 1·56 μmoles of APS in total volume of 1·2 ml.

Reaction	Added carrier (μmoles)	Rate of H_2 uptake (μmoles/hr)
(*a*) $SO_4^{--} \rightarrow H_2S$	—	0·1
	Cytochrome c_3, 0·31	2·1
	Methylviologen, 1	8·2
(*b*) APS → H_2S	—	1·0
	Cytochrome c_3, 0·42	1·9
	Methylviologen, 1	2·5
	Methylviologen, 0·1	2·7
	Cytochrome *c*, 0·11	1·0
(*c*) APS → SO_3^{--}	—	0·1
	Cytochrome c_3, 4·5	0·7
	Methylviologen, 1	1·3

Oxidation of Cytochrome c_3 *by APS in the Extracts*

In living cells of *Desulphovibrio*, it was found spectroscopically that cytochrome c_3 was reduced by the addition of hydrogen or formate and oxidized by the addition of sulphate, sulphite or thiosulphate (Ishimoto *et al.*, 1954). Similar experiments have now been carried out with the cell-free extracts.

The cytochrome c_3 in the extracts was reduced with gaseous hydrogen in Thunberg tubes. By this reduction the α-band at 553 mμ appeared, which was observed by a microspectroscope. The tubes were then evacuated and sulphate, sulphate plus ATP, APS, sulphite or thiosulphate was added from the hollow stoppers. At room temperature, the absorption band was weakened in 10 min in the tubes of sulphate plus ATP and APS, but only slightly in the sulphite tube even in 2 hr and no change was observed in the others as well as in the control tube without any addition. The results can be conceived to indicate the participation of cytochrome c_3 in the reduction of APS. The little change when sulphite and thiosulphate were added may depend on the considerably low potential of the systems compared with the potential of the cytochrome c_3 (E_0': $-0{\cdot}205$ V (Postgate, 1956)).

From the results of these experiments, the reductase in the extracts of *Desulphovibrio* can be regarded to play the role of the terminal oxidase of cytochrome c_3 just like the role of cytochrome oxidase in the aerobic systems.

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CYTOCHROME c_3

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The cytochrome known as c_3 is a pigment of one of the sulphate-reducing bacteria. It is one of the few bacterial cytochromes to have been obtained as yet as a single pure substance, and is unusual in being among the first cytochromes to be observed in obligately anaerobic organisms. The present contribution is a review of knowledge of its character and function, and is preceded by a brief account of the relevant characters of the bacteria in which it is found.

THE SULPHATE-REDUCING BACTERIA

The writer has recently reviewed several aspects of the physiology and taxonomy of these bacteria (Postgate, 1958, 1959) and the reader is referred to these reviews for documentation of most of the statements made herein.

These bacteria constitute a biochemical group of microbes which use the oxygen atoms of the sulphate ion as terminal electron acceptors for respiration in place of the free oxygen gas used by ordinary aerobic organisms. Sulphide is formed as a by-product of respiration; a typical metabolic reaction is:

$$2CH_3 \cdot CHOH \cdot COO^- + SO_4^{--} \rightleftharpoons 2CH_3 \cdot COO^- + 2H_2O + 2CO_2 + S^{--} \qquad (1)$$

Reaction (1) is almost universal throughout the group. Though species of sulphate-reducing bacteria able to utilize acetate have been reported, they are not well authenticated, and all the better-known species oxidize their carbon compounds down to a fatty acid level of oxidation only. In this character they might be regarded as anaerobic acetic acid bacteria.

They have also biochemical analogies to the nitrate-reducing bacteria, which can utilize the oxygen atoms of nitrate in place of free oxygen for respiration, but are distinguished from this group in being very exacting anaerobes. None of them grows in the presence of oxygen, and its absence is usually insufficient to ensure growth; they require an environment of negative E_h to start multiplication. In spite of their highly anaerobic character, their metabolism resembles that of an aerobe or a nitrate-reducing organism rather than that of an ordinary anaerobe because it is oxidative rather than fermentative. This point has been amplified by the author elsewhere (Postgate, 1958).

The authenticated species within this group of bacteria are three: *Clostridium nigrificans* (earlier known as *Desulphovibrio thermodesulphuricans*) a spore-forming thermophilic organism; *Desulphovibrio orientis*, a spore-forming mesophilic species; *Desulphovibrio desulphuricans*, a second mesophilic species which has salt-tolerant marine variants sometimes called *Desulphovibrio aestuarii*. It is the last species, *D. desulphuricans* and its marine variant, that contain cytochrome c_3.

D. desulphuricans is the sulphate-reducing bacterium that is most commonly encountered; it is widespread in soils, waters, sewage, industrial effluents and the rumens of sheep; in fact with proper techniques it can be detected in almost any aqueous environment, though it only multiplies when the oxygen tension is zero and the activities of other microbes have reduced the E_h to about $-0{\cdot}1$ V. It is of considerable importance in corrosion, pollution, oil technology, formation of sulphur and certain mineral deposits and in a variety of other economic spheres (these matters were also mentioned by Postgate, 1959) and it has been studied in the laboratory to a much greater extent than have the other two species. Its physiology has been discussed in the reviews already cited; for the present purposes, three biochemical properties need to be emphasized.

1. In addition to conducting reactions of the type given in equation (1), it contains a hydrogenase system enabling it to reduce sulphate in hydrogen:

$$4H_2 + SO_4^{--} \rightleftharpoons 4H_2O + S^{--} \qquad (2)$$

2. It can grow with and reduce a variety of partly reduced sulphur anions in place of sulphate. Examples are sulphite, thiosulphate, tetrathionate, dithionite; even colloidal sulphur. Of these only sulphite is a true intermediate in ordinary sulphate reduction, this fact having been demonstrated using labelled substrates.
3. Just as certain strains of aerobic bacteria can grow without air, so certain strains of *D. desulphuricans* can grow without sulphate, but such 'sulphate free' growth only takes place if pyruvate is present. Ordinarily pyruvate is metabolised by reaction (3) analogous to (1) above.

$$4CH_3{\cdot}CO{\cdot}COO^- + SO_4^{--} \rightleftharpoons 4CH_3{\cdot}COO^- + 4CO_2 + S^{--} \qquad (3)$$

but if sulphate is absent the true fermentation (4), yielding gaseous hydrogen, occurs:

$$CH_3{\cdot}CO{\cdot}COO^- + H_2O \rightleftharpoons CH_3{\cdot}COO^- + CO_2 + H_2 \qquad (4)$$

PROPERTIES OF CYTOCHROME C_3

The properties of cytochrome c_3 (Postgate, 1956) and its associated pigment desulphoviridin have been reviewed briefly with an appropriate bibliography (Postgate, 1959).

Once the bacteria have grown (and their cultivation has been facilitated in recent years) cytochrome c_3 may be prepared in a high state of purity very simply. A procedure occupying three to four days is given below:

Day 1: Squirt a cream of living or vacuum-dried *D. desulphuricans* (about 30 g dry wt) into 1 l. of boiling KH_2PO_4 (0·5%, pH 7). Allow to boil for 3 min, cool and add $(NH_4)_2SO_4$ to 0·75 saturation. Centrifuge (15 min at 2500 rev/min) and collect 'internatant' red solution by pouring carefully through glass wool. Adjust to pH 2·6 with 2 N H_2SO_4 (check pH after 60 min) and store overnight at 4°C.

Day 2: Centrifuge (20 min at 2500 rev/min), dissolve residue in 0·25 N NH_4OH, and store as fraction *A*. Adjust supernatant fluid to pH 2·6, stir in Whatman 'Standard' powdered cellulose and pour into a chromatography tube to form a cellulose column (about 70 ml wet cellulose). Pass whole volume of effluent once through the column; do not wash the column but elute red material directly with 0·25 N NH_4OH. Combine eluate with fraction *A* and dialyse overnight.

Day 3: Stir dialysed red solution with fine Amberlite IRC-50 (3–20 min settling fraction) in the ammonium form. Pour to form a column (about 20 ml wet resin), wash pink resin with distilled water (20 vol; discard denatured material which is not held by the resin) and elute with 0·25 N NH_4OH. Dialyse and freeze dry product. Yield: 15–20 mg native cytochrome c_3.

The product is at least 94% pure and is a deep red powder. It is the ammonium salt of cytochrome c_3 and has the following properties (Table 1).

TABLE 1. PROPERTIES OF CYTOCHROME c_3

Fe content (%)	0·91 ± 0·01
E_0' (V, at pH 7)	−0·204 ± 0·005
Iso-electric pH	10·5
Specific extinction coefficient ($E_{1\text{cm}}^{1\%}$) for ferro form, at 553 mμ	4·0
Mol. wt.	About 12,000, and not less than 10,200

These data indicate a molecular weight approximating to that of muscle cytochrome *c* and suggest that the molecule possesses two haematin groups instead of one. The haematin groups are stable to boiling and to acid acetone but are removed by reagents which split thio-ethers (acid silver sulphate, mercury amalgam). The degradation products are spectroscopically closely similar to those obtained from cytochrome *c* (Table 2).

TABLE 2. ABSORPTION SPECTRA OF CYTOCHROMES *c* AND c_3

(All wavelengths are in mμ)

Absorption peaks of:	Cytochrome *c*			Cytochrome c_3		
Native protein (ferro)	418	520	550·4	419	525·2	553·2
Nitroso-protein (ferro)		531·1	563·4		532·5	563·5
CO-protein (ferro)	415	531·5	564·5	415	530	565
'Pyridine haemochrome'	415	521·8	550·6	413	521	551·8
'Porphyrin *c* or c_3' (conc. HCl)			553			554
Haemin	390*			391		
Chlorin formed with Na/Hg			643			643
Porphyrin with Na/Hg (in dioxane)	402 497	530·2 566·5	621·5†	402 496·5	529·8 556·8	621·5

* Haematohaemin † Mesoporphyrin

The amino acids present in hydrolysates of cytochrome c_3 have been studied qualitatively by paper chromatography: those present are cystine, histidine, lysine, arginine, methionine, phenylalanine, the leucines. Valine was doubtful; diaminopimelic acid, hydroxyproline, and glutamine were absent; the native protein gave no reaction for tryptophan. Quantitative studies have not been undertaken. Compared with control hydrolysates of commercial cytochrome *c*, hydrolysates of c_3 appeared to contain greater quantities of histidine and cystine and less of threonine and lysine; in addition, the commercial cytochrome *c* (Sigma) contained tryptophan, but otherwise the amino-acid patterns were similar.

These observations lead to the conclusion that cytochrome c_3 is a protein of the same general character and size as muscle cytochrome *c*, but having two haem residues in the molecule instead of one. These haem groups are bound to the apo-protein by thio-ether linkages as well as by co-ordination to their central iron atom.

Cytochrome c_3 is associated in the cells with a soluble green pigment called desulphoviridin, which has a strong absorption band at about 630 mμ. This is not cytochrome a_2; it undergoes no oxido-reduction reactions and decomposes readily with acids, alkalis or heat to yield not a haem but a free porphyrin. The latter has a strange spectrum recalling most closely that to be expected for a water-soluble chlorin; it is blue-green in colour and is rapidly photo-oxidized to a colourless derivative.

Desulphoviridin is thus a kind of porphyroprotein. It has not been purified, but concentrates freed of other strongly absorbing materials show an α-band at 632 mμ, β-band at 585 mμ, and an unsymmetrical Soret peak at 411 mμ. The free prosthetic group, after chromatography on 'Florisil', is an indicator and shows the following absorption bands in aqueous solution (the heights and positions of the double Soret peak in acid solutions are not influenced by further acidification):

Neutral pH: 404 mμ, 551 mμ, 594·5 mμ
Acid pH: 385 mμ, 404 mμ, 575 mμ, 613·5 mμ

The function of desulphoviridin is unknown. It is associated with various enzymically active fractions of the bacteria but is identical with none.

METABOLIC FUNCTION OF CYTOCHROME C_3

Japanese work on this question was summarized by Ishimoto, Kondo, Kamayama, Yagi and Shiraki (1958) and a brief but wider review was given by Postgate (1959). A summary of the present position is given below.

Thiosulphate or Tetrathionate Reduction

Intracellular cytochrome c_3 becomes partly oxidized when bacteria are incubated anaerobically with these substrates. Soluble reductase preparations which reduce these substrates in hydrogen have been prepared from the bacteria; the reactions are accelerated by purified cytochrome c_3. Benzyl or methylviologen will act as an artificial electron carrier in place of cytochrome c_3.

Dismutation of Formate or Pyruvate

Cytochrome c_3 accelerates the action of enzyme preparations which dismute formate to hydrogen and CO_2, but it has no influence on the dismutation of pyruvate to acetate, CO_2 and H_2 (reaction 4).

Reduction of Colloidal Sulphur, Nitrite, Hydroxylamine, Oxygen

All these substrates are reduced in hydrogen by cell suspensions or enzyme preparations. With enzyme preparations the reactions are accelerated by cytochrome c_3. These reductions, are, however, biochemical artefacts in the sense that they result from direct chemical oxidation of ferro-cytochrome c_3 by the substrate and no specific reductase is involved. They can be represented schematically as:

$$H_2 \xleftrightarrow{\text{enzyme (hydrogenase)}} \text{cyt}\,.\,c_3 \xleftrightarrow{\text{spontaneous}} NH_2OH$$

Dyestuffs such as benzyl- or methylviologen will replace cytochrome c_3 in such reactions. Reactions of this kind may be expected to occur in any system involving low-potential electron transport enzymes; the possibly 'artificial' character of certain accepted steps in the reduction of nitrate was pointed out by Senez and Pichinoty (1958).

Reduction of Sulphite

Oxidation of c_3 by sulphite can be observed spectroscopically in intact bacteria, but the enzymological evidence for participation of cytochrome c_3 in sulphite reduction is inconclusive. Some enzyme preparations show small stimulations of sulphite reduction when extra cytochrome c_3 is added. Recent work by the author has shown that the sulphite reductase system is complex, as indicated by the following results.

Cells of *Desulphovibrio desulphuricans* (Hildenborough) grown in continuous culture were disrupted by treatment with liquid N_2 (Moses, 1955) and treated with deoxyribonuclease (to keep the preparation liquid). Centrifugation yielded a red particulate fraction having most of the sulphite reductase activity (together with much bound cytochrome c_3) and a supernatant fraction without which the sulphite reductase activity of the particulate preparation was negligible unless benzylviologen were added. The particles then showed 1·5 to 3% of the sulphite reductase activity of the original cells; the liquid fraction had only slight reductase activity though hydrogenase alone was plentiful. The liquid 'co-sulphite reductase' could be further fractionated into two heat-labile proteins (one soluble in 36% ammonium sulphate, the other not) and a dialysable factor that could be recovered by freeze drying the dialysate. Addition of cytochrome c_3 to the complete preparation (particles and soluble fraction) did not influence the rate of reduction of sulphite at all (there is probably sufficient cytochrome c_3 bound with the particles) even if cytochrome c_3 had previously been removed from the soluble fraction by ion-exchange. Complete enzyme preparations were stimulated further by benzylviologen but not by diphosphopyridine nucleotide, triphosphopyridine nucleotide, coenzyme A, α-lipoic acid, hypoxanthine, methyl-1:4-naphthoquinone, pyridoxal phosphate, adenosine-3′-phosphate, pyruvate, succinate, cysteine, glutathione, a concentrate of desulphoviridin, or by salts of the following metals: Mo, V, Mn, Co, Ni, Mg, Zn or Ca. Modest stimulation was observed with adenosine triphosphate (ATP) and acetyl phosphate and a slight effect occurred with ferrous ions; these materials did not, however, replace the dialysable component referred to earlier.

Some quantitative data obtained with particulate sulphite reductase preparations are given in Table 3.

These observations show that the sulphite reductase system involves several components:

1. hydrogenase
2. thermolabile protein A
3. thermolabile protein B
4. cytochrome c_3
5. a dialysable co-factor
6. a sulphite reductase.

TABLE 3. ACTIVITY OF SULPHITE REDUCTASE PREPARATIONS

Experiments were conducted in conventional Warburg manometers under hydrogen gas at 37°C in 0·5% KH_2PO_4 (pH 6·9) with $CdCl_2$ in centre well. Negative Q_{H_2} values for Na_2SO_3 recorded (in μl H_2 absorbed/mg N/hr) from five experiments.

Preparations	Hydrogen absorbed (μl/mg N/hr)
(1) Intact bacteria	5,400
(2) Whole enzyme preparation	157
Soluble fraction	25†
Particulate fraction	11†
(3) Particulate fraction	16
Particulate fraction + *A**	14·5
Particulate fraction + *B**	15
Particulate fraction + *A* + *B*	62
(4) Particulate fraction	5·8
Particulate fraction + benzylviologen (0·36 μmole/ml)	132
(5) Whole preparation	32
Whole preparation + ~PO$_4$‡	87

* *A* and *B* represent further fractions from the soluble fraction, one (*A*) soluble in 0·5 saturated $(NH_4)_2SO_4$, the other (*B*) not.

† *Q* values of fractions refer to the N content of the original unfractionated material.

‡ ATP (sodium salt) and acetyl phosphate (lithium salt) 70 μg of each/ml.

In addition, 'energy-rich' phosphate may be used in the primary activation of sulphite.

Reduction of Sulphate

The most obvious function of cytochrome c_3, that of being a co-factor in the reduction of sulphate, is the furthest from being established experimentally (Note 1). The reasons for believing that cytochrome c_3 has such a function are four:

1. It is *a priori* likely that anaerobic sulphate reduction would involve an electron transport system analogous to the reduction of oxygen or nitrate. Cytochrome c_3 is present in all strains of *D. desulphuricans* and haematins are to be found in the other well-authenticated sulphate-reducing bacteria; these facts suggest that haematins are universally involved in this biochemical process.
2. Anaerobic oxidation of intracellular cytochrome c_3 by sulphate can be observed with proper precautions. This reaction is inhibited by known specific inhibitors of sulphate reduction (selenate, monofluorophosphate).
3. Cells starved of inorganic iron are deficient in cytochrome c_3, and in ability to reduce sulphate. They can, in fact, only grow by the fermentative reaction (4) above and hence are only obtainable in pyruvate media.

4. Sulphite, or its biochemical equivalent, is the one established intermediate in sulphate reduction. Since there is some reason to believe that c_3 is concerned in the functioning of the sulphite reductase, it follows that it must equally be involved in the reduction of sulphate.

It is reasonable to conclude that cytochrome c_3 is the sole haematin compound among several electron transport enzymes of a conventional character linking sulphate reduction to dissimilation processes in *D. desulphuricans*. In aerobic organisms, cytochrome c_3 is replaced by the cytochromes of the *a*, *b* and *c* types (or an analogue of this system) and the function of the sulphate reductase is taken over by cytochrome oxidase.

Since this contribution was prepared, Ishimoto and colleagues have obtained a cell-free sulphate reductase which requires adenosine triphosphate (ATP). The reduction of sulphate by this preparation is stimulated by addition of cytochrome c_3. These experiments are mentioned by Egami, Ishimoto and Taniguchi in their contribution to this publication (see p. 404).

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DISCUSSION

Nature and Properties of Cytochrome c$_3$

Crystallization of cytochrome c$_3$

Horio: Kamen and I, very recently, succeeded in crystallization of cytochrome c_3 from dried cells of *Desulphovibrio desulphuricans* which had been kindly sent by Postgate.

To our regret, the general properties have not yet been studied.

However, the story about the native and modified forms is the same in the case of cytochrome c_3 as it is with typical cytochrome *c*'s.

As indicated by Postgate, this bacterial cytochrome is the first one extracted from bacteria which has a high iso-electric point. This property makes its purification on Amberlite CG-50 quite effective, unlike the *Pseudomonas* cytochromes.

With the lyophilized cells, most of cytochrome c_3 is easily extractable with the use of 0·1 M sodium citrate at a neutral pH, and it can be chromatographed with the use of 0·15 M (as NH_4^+) ammonium phosphate buffer (pH 7·0). In addition to that fraction which is not adsorbed on the resin, cytochrome c_3 is chromatographed into 4 fractions. One of the fractions is eluted with 0·05 N ammonia after the chromatography with the buffer.

From one of the fractions, cytochrome c_3 has been crystallized. The other fractions have been found to be artificially made by treating the crystalline sample under drastic conditions; high and low pH, and boiling. Therefore, we can say that the crystallized sample is most plausibly native, and that cytochrome c_3 is fairly unstable to these treatments.

On the Structural Significance of Two Haem Groups/Molecule in Cytochrome c_3

HORIO. As I said in the previous session, one of the modified cytochrome *c*'s exists in a dimer form; the $S_{20,w}$ and $D_{20,w}$ of the modified cytochrome *c* gave its mol. wt. to be 24,400. On the other hand, Theorell reported that tryptic cytochrome *c* (mol. wt. approximately 2,000) can form a polymer. But the polymer can be changed into the original monomer (maybe containing histidine) if histidine is added to the polymer sample.

Of course, I do not attempt to say that the modified cytochrome c_3 is formed in the same way, because its iron content is very high compared with other cytochrome *c*'s.

But at least, if it is assumed that cytochrome *c* can occur in a state in which the imidazole radical (or radicals) is dissociated from haem iron, then two molecules in such a state could form a dimer. Of course, this is only one of the possibilities.

MORTON: Heart-muscle cytochrome *c* may be heated to about 100°C in dilute salt solutions at pH 7 and, on cooling, shows the same absorption spectrum as before heating. If cytochrome c_3 can exist as a dimer it might be expected that the absorption spectrum would change on boiling. Is there any evidence available on this point?

POSTGATE: The material retains its enzymic activity and chromatographic properties after boiling at pH 7 in dilute salt solutions. I do not know if the spectrum changes, but the solution does not obviously decolorize.

HENDERSON: Knowledge of the *n* value of cytochrome c_3 would be useful. This would indicate whether there is interaction between the haems, i.e. if so $n = 2$, if not $n = 1$, and still in the latter case it could have two haems/molecule or aggregate. This would help considerably in elucidation of electron transport problems (cf. Shack and Clark: *J. biol. Chem.* **171,** 143, 1947).

Amino Acid Composition and Absorption Spectrum of Cytochrome c_3

EGAMI: Takahashi (Takahashi, Titani and Minakami, *J. Biochem. Tokyo*, **46**, 1323, 1959) in my laboratory in Tokyo carried out the amino acid analysis of cytochrome c_3. He found that it contains no tyrosine and just twice as many (6) residues of histidine as mammalian cytochrome *c*. The former finding is consistent with the observation that it has no absorption at 280 mμ and the latter seems to be consistent with the fact that it contains just twice the haem iron.

DRABKIN: I believe that the mystery of the missing band at 280 mμ of cytochrome c_3 is now somewhat dispelled, since we have now seen the absorption spectrum of the oxidized form. If you will turn to Fig. 5 of my paper (p. 150) you will find that the spectrum of ferriprotohaemin dicyanide is very similar indeed to that of ferricytochrome c_3. There is no distinct maximum, but certainly definite absorption in this region. The analysis discloses the presence of my band No. 8 at approximately 280 mμ, masked in this particular case, and perhaps also in cytochrome c_3.

Functional Aspects of Cytochrome c_3

On the Functional Significance of Two Haem Groups/Molecule in Cytochrome c_3

TRUDINGER: *Desulphovibrio desulphuricans* is unusual in being one of the few known bacteria containing a single haemoprotein. Moreover this protein contains two haem groups/molecule as distinct from the usual case of one haem group/protein molecule in '*c* type' cytochromes. Would any of the workers involved with cytochrome c_3 care to comment on a possible significance in this coincidence?

POSTGATE: I cannot suggest any specific reason for this haematin being bifunctional, but the haem residues appear to be identical in character and linkage, and the organism seems to be the only recorded organism to have one, and not more than one, cytochrome.

KAMEN: An interesting situation exists in the energetics of the electron transport system of *Desulphovibrio*. The potential difference between hydrogen and the terminal sulphur compounds for reduction to sulphide is insufficient for production of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate with transfer of one electron. Even with the most extreme assumptions about concentration of redox complex involved, there is barely sufficient energy available. However, two electrons must be transferred to reduce SO_4^{--} to SO_3^{--}. Perhaps the double haem character of cytochrome c_3 is attached to this requirement. There are no haematin compounds other than cytochrome c_3 present, whereas in other systems there are at least two and usually more species of haematin compounds present. Perhaps the necessity of multiple haem groups is satisfied in *Desulphovibrio* by putting two haems in one protein.

In relation to the use of potential difference for these calculations, one can state that standard potentials cannot be applied directly but within a range of ± 100 mV concentration effects only partially invalidate considerations based on such potentials. An excellent discussion of this matter is given by Hill in his review of oxidation-reduction potentials (*Handbuch der Pflanzenphysiologie 5*, 1960)

Function of Cytochrome c₃ *in Hydroxylamine Reduction*

EGAMI: In relation to Postgate's findings, we found that hydroxylamine reduction by cytochrome c_3 was enhanced by addition of Mn^{++}, in the same way as was the reduction by the bacterial hydroxylamine reductase.

As to be expected from thermodynamical data, our experiments show that there is no requirement for activation of sulphite by adenosine triphosphate or other activating compounds before reduction.

POSTGATE: It is quite possible that our sulphite contained some sulphate, and that the apparent effect of ATP was due to its permitting reduction of sulphate in addition to the sulphite.

The Occurrence of Cytochrome c₃ *in* Desulphovibrio Desulphuricans

ESTABROOK: It has been repeatedly stated that cytochrome c_3 is the *only* cytochrome in this organism. What is the evidence to support this claim?

POSTGATE: The spectrum of intact organism shows no splitting in liquid nitrogen, nor does the spectrum of extracted material. All extraction procedures yield cytochrome c_3 in the supernatant and residue and nothing else; the total haematin present as pyridine haemochrome is quantitatively similar to the ordinary cytochrome c_3 content.

KAMEN: Repeated efforts to extract haem compounds other than cytochrome c_3 have failed. Residues from all extraction procedures show only bound cytochrome c_3.

Evolutionary Aspects of the Sulphate-reducing Bacteria and of Cytochrome c₃

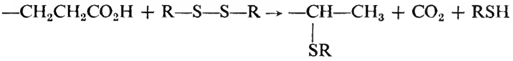
LEGGE: Could Postgate comment on the possible evolutionary position occupied by the sulphate reducing bacteria?

POSTGATE: Thode and his colleagues in Canada (*Geochim. cosmochim. Acta* 3, 235, 1953) have shown that the microbial sulphur cycle leads to partial fractionation of the sulphur isotopes. Minerals such as sulphur or metal sulphides formed by way of microbial action are enriched with the light natural isotope and the residual sulphate becomes enriched with the heavier natural isotope. The major contribution to this fractionation is made by the sulphate-reducing bacteria.

By examining sulphur-bearing deposits of various geological ages they have detected such fractionation as far back as 8×10^8 years ago, from which it seems likely that massive sulphate-reduction occurred as far back as then. It seems that all dissimilatory sulphate-reducers today are obligate anaerobes, and this is perfectly consistent with the current belief that 8×10^8 years ago the earth's atmosphere was anaerobic. If at that time they required haematins of the cytochrome type, as they do today, it seems reasonable to suppose that these cytochromes represent the evolutionary precursors of the more complex aerobic cytochrome systems that are widespread today.

Kamen: Even if one accepts the possibility that the sulphate reducers represent a primitive type of haematin system the question still remains how the haematin compounds could be produced in the absence of oxygen. We do not know as yet how strict anaerobes synthesize the haem moiety. The glycine-succinic-porphobilinogen sequence appears to be based on an oxygen requirement. The possibility that some sort of primitive haem existed in a pre-formed state prior to the development of the cytochromes seems very far-fetched but cannot, of course, be ruled out.

Lemberg: It appears likely from geochemical considerations that organisms using sulphate as oxidant may have been evolved early. One may perhaps put up here an interesting speculation. Cytochromes *c* appear to be extraordinarily complex haemoproteins and still are found in these (probably) early organisms. One may perhaps bring together the facts that oxygen was absent in the early earth atmosphere, that oxygen is known to be required for the oxidative decarboxylation of propionic acid to vinyl side chains in protohaem formation, and that cysteine-thioether linkages are found in cytochromes *c*, by assuming that oxidative decarboxylation of the propionic acid side chains by oxygen was preceded by one involving protein-cystine, leading simultaneously to thioether linkage formation, thus:



$$\mathrm{—CH_2CH_2CO_2H + R—S—S—R \rightarrow —\underset{\substack{|\\ SR}}{CH}—CH_3 + CO_2 + RSH}$$

Are these catalase-free organisms sensitive to oxygen, and is anything known of their pyruvate metabolism?

Postgate: The organism is totally deficient in catalase; its metabolism is stopped by oxygen but it is not killed even after several hours in air. Its pyruvate metabolism is phosphoclastic, yielding acetyl phosphate, CO_2 and either free hydrogen or ethanol.

Falk: The question of the synthesis of protohaem by some anaerobes is a very interesting one. In animal tissues, oxygen is *required* in the biosynthetic pathway for the formation of succinyl-coenzyme A, and again for the conversion of two propionic acid groups to vinyl groups: all the remaining steps can be performed anaerobically. It has been found recently (Falk *et al.*, *Nature*, *Lond.* **184**, 1217, 1959) that for protoporphyrin synthesis in chicken erythrocytes *in vitro* there is a sharp optimal oxygen tension at about 0·07 atmospheres; oxygen tensions higher than this are increasingly *inhibitory* and at the 0·2 atm. of sea-level air the inhibition is already some 25%. We have found that this inhibition by oxygen operates somewhere after porphobilinogen has been formed, and before uroporphyrin appears, and we are currently looking for the sensitive reaction. We feel that this inhibition is directly related to the adaptation of haemopoiesis to oxygen tension (e.g. altitude) and to such adaptations as those found in *Daphnia* and other Crustacea and invertebrates, though other factors also are no doubt involved.

It appears possible that this control is one of the factors in adaptive cytochrome synthesis in micro-organisms also; in fact it was the finding of Moss (*Aust. J. exp. Biol. med. Sci.* **34**, 395, 1956) that the synthesis of cytochromes by *Aerobactor aerogenes* shows an *optimum* against oxygen tension which led us to these studies. To return to the less common but definite anaerobic formation of protohaem by micro-organisms, the requirements, as different from animal tissues, are that the formation of succinyl coenzyme A and of vinyl-groups must occur anaerobically. As far as the latter is concerned, it is easy to postulate a dehydrogenase with a terminal acceptor other than oxygen. In a way, we have an analogy for this, though in a reductive, not an oxidative, dehydrogenase-type reaction, in the enzymic, non photo-catalytic conversion of protochlorophyll to chlorophyll in certain plants.

Kaziro: In connexion with the problem now under discussion, I would like to mention that there are some bacteria, not strictly anaerobic, which produce hydrogen peroxide as a metabolite. Since they have no catalase, the hydrogen peroxide accumulates in the medium as they proliferate.

It might be of some interest, as suggested now by Lemberg, to refer to a peculiar disturbance observed in the cases of congenital deficiency of catalase in man. I have had the opportunity of finding some cases of these deficiencies and to investigate them. In most of these cases a peculiar malignant inflammation over the oral region was observed which started from the root part of the teeth and resulted in severe progressive gangrenous ulcers. Often, the ulcer progressed to the jaw bone. The disturbance has been designated as Takahara's disease after the name of the first discoverer. The pathogenesis has so far been explained as the result of bacterial infections and the tissue disturbance is caused by a toxic action of hydrogen peroxide produced continuously by the infecting bacteria. These grow well at the gingiva, especially where gingiva and teeth root touch. In some cases, the infection was reported to have started from fissures in the tonsil. Among bacteria which are found in the infected gingiva are strains of *Lactobacillus*, *Streptococcus haemolyticus*, *Diplococcus pneumoniae* and others. They are known to produce hydrogen peroxide and have no catalase.

The above-mentioned abnormality has long been reported in Japan and so far in Japan only. It was found for the first time by Takahara in 1946 at the polyclinic of the Okayama University. Until now, we have had 35 cases of the abnormality originating from 16 families which have been described by various physicians in different places in Japan (see Nishimura *et al.*, *Science* **130**, 330, 1959).

THE ATYPICAL HAEMOPROTEIN OF PURPLE PHOTOSYNTHETIC BACTERIA

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INTRODUCTION

ALL PHOTOSYNTHETIC systems contain large amounts of haemoproteins bound to photoactive subcellular aggregates (Kamen, 1957). These haematin compounds are in many ways quite similar to those of the familiar respiratory systems and can be included in the categories known to biologists since the classic work of Keilin. However, there appears to be a novel type of haemoprotein which can be extracted in soluble form from all the common species of purple photosynthetic bacteria (Vernon and Kamen, 1954; Kamen and Vernon, 1955; Newton and Kamen, 1956; Bartsch and Kamen, 1958) and which cannot be placed in any single recognized class of haemoproteins, e.g. cytochromes, haemoglobins, catalases, etc. On the occasion of the present symposium, it is appropriate to summarize briefly our knowledge about this atypical haemoprotein.

GENERAL PROPERTIES

Distribution of the Atypical Haemoprotein ('RHP')

The first specimen to be noted and isolated was found in trichloroacetic acid extracts prepared from cell suspensions of the facultative photoheterotroph, *Rhodospirillum rubrum* (Vernon and Kamen, 1954). At first, the compound was thought, on spectroscopic grounds, to be a "pseudohaemoglobin'. In further studies, the revelation of its anomalous character created uncertainty mirrored in a series of progressively less definite names which culminated finally in the present noncommittal '*Rhodospirillum haem protein*', abbreviated to 'RHP'. We now customarily use 'RHP' to refer to all examples of the atypical haemoprotein, regardless of the species from which it may be obtained.

RHP has been found in representative species of all the presently-recognized genera of the purple non-sulphur bacteria (*Athiorhodaceae*) and in the only readily available species (strain D of the genus *Chromatium*) which is representative of the purple sulphur bacteria (*Thiorhodaceae*) (Kamen and Vernon, 1955). Preliminary attempts to detect RHP in other photosynthetic systems, such as green plants, algae and the green sulphur bacteria, have been unsuccessful to the present. If further studies, now in progress, should confirm that the occurrence of this unique haemoprotein is restricted to the purple bacteria, this result would occasion no great surprise. The same condition exists with regard to the chlorophyll and carotenoid components of the purple bacteria, relative to those of other photosynthetic tissues (Van Niel, 1944; Goodwin, 1955; Goodwin and Land, 1956*a*; Goodwin, 1956; Goodwin, Land and Sissins, 1956; Goodwin and Land, 1956*b*).

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It is possible that a haematin compound similar to RHP is present in a halotolerant nitrate-reducing *coccus* studied by Taniguchi, Asano, Iida, Kono, Ohmachi and Egami (1958). A soluble fraction obtained from cell suspensions of this micro-organism contains an enzyme active as a hydroxylamine reductase, which exhibits absorption spectra and chemical properties closely similar to those of RHP (see discussion in a later section).

The fact that RHP occurs in the purple photosynthetic bacteria, and possibly in the nitrate-reducing bacterium mentioned above, points to the desirability of searching for RHP among bacterial species which are chemosynthetic analogues of the photosynthetic bacteria, e.g. sulphate-reducers, sulphur oxidizers, ammonia and hydrogen oxidizers, nitrate-reducers, etc.

Many studies on the absorption properties of bacterial suspensions using the elegant methods of dynamic spectrophotometry developed by Chance (1954), Duysens (1954) and others have revealed the existence of spectroscopic entities among a variety of bacterial species (*Bacillus subtilis*, *Proteus vulgaris*, *Achromobacter fischeri*, *Staphylococcus albus*, *etc*.) which appear to resemble RHP closely (Smith, 1954). These pigments appear to function as terminal oxidases, and the term, 'cytochrome *o*', has been suggested to denote them as a class (Castor and Chance, 1959). In the particular case of *R. rubrum*, isolated RHP and the cytochrome *o* moiety are so much alike spectroscopically, there is strong temptation to equate them and to consider RHP as a prototype for the cytochrome *o* class. We shall return to this point later.

Physical Properties

RHP has been isolated in varying degrees of purity from all the purple photosynthetic bacteria, but in only two cases has it been obtained sufficiently pure and in such amounts as to permit extensive investigation of its physical and chemical properties. These are the original RHP of *R. rubrum* and that of the strict photoanaerobe, *Chromatium* (strain D). Salient physical properties of these two preparations are listed in Table 1. The references cited in this table contain detailed descriptions of methods used. It will be seen, not only in connection with these particular properties but with others discussed later, that RHP's vary somewhat in detailed physical nature. However, the variation is not more than that encountered in other established categories of haematin compounds, such as cytochromes of the *c* type, *b* type, haemoglobins, etc.

TABLE 1. PHYSICAL PROPERTIES OF RHP*

(Cf. Newton and Kamen, 1956; Bartsch and Kamen, 1958)

Property	*R. rubrum*	*Chromatium*
Isoelectric point (pH)	4·43	5·5 ± 0·1
$S_{20,w}$†	$2·7 \times 10^{-13}$	$3·2 \times 10^{-13}$
$D_{20,w}$†	$8·65 \times 10^{-7}$	$7·5 \times 10^{-7}$
$\overline{V}$ (cm³/g)	0·731	0·706
μ† (pH 5·0, ionic strength = 0·1)‡	+6·8	+5·4
Mol. wt.	26,000 (±2,000)	36,000 (±3,000)
E_0' (pH 7·0)	−0·008 V§	−0·005 V§

* Values in this table corrected in proof; see Horio and Kamen, 1960.

† C.G.S. units.

‡ Electrophoretic mobilities at other pH and ionic strengths are omitted. See original references cited above.

§ Electrometric titration shows that oxidation-reduction of RHP is reversible and involves a one-electron change.

Chemical Properties

The chemical behaviour of RHP has been studied mainly using the *R. rubrum* preparation, but recent studies (Bartsch and Kamen, 1960) have shown that results obtained with this preparation are duplicated in every detail using the *Chromatium* RHP. We shall summarize here the chemical properties most relevant for the present discussion.

RHP is soluble in water. It appears to have minimal solubility below pH 5, as is expected from its isoelectric point. In general, it salts out with ammonium sulphate at concentrations which differ slightly from those effective with the corresponding cytochrome *c* protein obtained from the same source. However, RHP shows solubility behaviour so much like cytochrome *c* that it is advantageous to devise extraction procedures which minimize contamination with cytochrome *c* before commencing extensive fractionation of RHP (Bartsch and Kamen, 1958).

RHP resembles mammalian cytochrome *c* in heat stability and resistance to denaturation by acid or base. However, at strongly alkaline pH (greater than 11), RHP undergoes a reversible denaturation in which it assumes the spectrochemical form of a haemochrome like cytochrome *c*, with characteristic triply-banded spectra in oxidized and reduced forms. The original myoglobin-like spectra are obtained when the pH is brought back to neutrality.

Unlike cytochrome *c*, RHP is rapidly auto-oxidizable. It can be oxidized and reduced reversibly using the reagents commonly employed for this purpose in chemical manipulations of haematin compounds. The alkaline haemochrome form of RHP can also be oxidized and reduced reversibly. However, in the presence of a detergent such as lauryl sulphate, repeated cycles of oxidation and reduction at $pH > 11{\cdot}8$ result in progressive loss of haem. This result is not observed at pH 7. The irreversible oxidative degradation in alkali is reminiscent of the behaviour of haemoglobin in the presence of detergents at physiological pH.

If RHP, while still partially denatured in dilute alkali and in the presence of lauryl sulphate, is incubated with 4-methylimidazole, it forms a typical haemochrome. Denaturation with strong alkali is required before ligands such as cyanide, pyridine, fluoride and azide will bind the central iron atom of RHP.

The prosthetic group cannot be split by cold acid–acetone, as in myoglobin, haemoglobin, or cytochrome *b*; reductive cleavage with sodium amalgam, strong acid and heat, or fission with silver salts and acid (Paul, 1951) are required as in cytochrome *c*. The products obtained by the sodium amalgam procedure (Davenport, 1952) are identical with those derived from cytochrome *c*. The pyridine and cyanide haemochromes derived after denaturation in strong alkali are identical spectroscopically with those of cytochrome *c* (Vernon and Kamen, 1954). Hence, it is likely that the prosthetic group of RHP is identical in type and nature of binding with that of the haem in cytochrome *c*, that is, the haem group is bonded by saturated (thioether) linkages between the vinyl side chains of the haem moiety and cysteine residues of the protein.

RHP is most peculiar in its response to reagents which normally form reversible addition products with haem. Thus, it does not react at neutral pH with H_2S, O_2, NO, NaN_3, NaCN and 4-methylimidazole, either in the reduced or oxidized form. In this respect, it resembles cytochrome *c* or cytochrome *b*. However, it will bind carbon monoxide reversibly in the reduced form to give a typical haemochrome, which is extremely light-sensitive. If the pH is kept below 4, the carbon monoxide complex is very light-stable.

The chemistry of RHP can be understood on the basis that it is a variant of cytochrome *c*, capable of reversible denaturation in varying degree, as evidenced by its response to various ligands. The particular variation introduced in RHP may be assumed to be a coiling of the peptide chains, so that one or both of the usual basic co-ordination groups of the protein cannot bind to the extraplanar valences of the central iron atom.

Falk and Nyholm (1957) have presented correlations between electrochemical potential, spectra, mode of binding and substituents on the

β-carbons of the pyrrole moieties of porphyrins and metalloporphyrins. These support the suggestion (Bartsch and Kamen, 1958) that the protein presents an electronegative group, such as carboxyl, to the central iron atom of the haem (see also, Williams, this Symposium, p. 41) and that it is this functional grouping (possibly as part of a long chain) which prevents approach of a basic co-ordinating group. It will be of great interest to attempt isolation of a haem peptide from RHP and if successful, to ascertain its structure.

Immunochemical Properties

All of the haemoproteins, including the RHP type, derived from either *R. rubrum* or *Chromatium*, can function as antigens, evoking characteristic antibody synthesis in rabbit sera. The antibody against any one haemoprotein, when tested by the Ouchterlony technique, by precipitation, by complement fixation, etc., does not appear to interact with any other haemoprotein, whether of a different type from the same bacterium or of the same type from a different bacterium. For example, the anti-RHP of *Chromatium* will not interact with the *R. rubrum* RHP, nor with cytochrome *c* from either *Chromatium* or *R. rubrum*. Thus, there are strain differences in RHP from different sources, just as there are in the haemoglobins and myoglobins.

The antigenic properties of RHP preparations afford a means for applying immunochemical techniques as aids in structure determination, and also as a basis for monitoring denaturation during extraction.

Spectroscopic Properties

In Figs. 1 to 5, characteristic features of the absorption spectra under various conditions are exhibited. The main properties may be summarized as follows.

1. Spectra of the oxidized and reduced forms (Figs. 1–3) resemble those for a myoglobin pigment, except for a general shift of all absorption maxima toward shorter wavelengths than those found for myoglobin or peroxidase. This shift accords with the apparent mesohaem character of the prosthetic group of RHP.
2. There is a characteristic haematin band at 630–640 mμ which disappears on reduction and reappears on oxidation. This band is used for preliminary monitoring of extracts for the presence of RHP.
3. The Soret band of the reduced form is complex, showing a persistent shoulder some 5–10 mμ on the long wavelength side of the maximum absorption at 423–426 mμ. There appears to be a more pronounced shoulder in the *R. rubrum* compound (Fig. 1) than in the *Chromatium* compound (Fig. 3).
4. Carbon monoxide forms a complex with reduced RHP, showing a typical haemochrome spectrum (Fig. 5). As compared with unreacted reduced RHP, the Soret peak of the complex RHP sharpens, intensifies, and

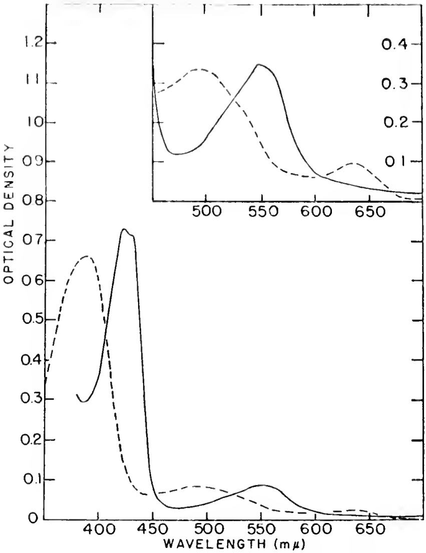

FIG. 1. Spectrum of oxidized (broken line) and reduced (solid line) *R. rubrum* RHP at pH 7. For the main curves, 0·66 × 10^{-2} μmoles/ml, and for the inset curves 2·7 × 10^{-2} μmole/ml in M/15 phosphate were used. (Reproduced by permission of the *Journal of Biological Chemistry*.)

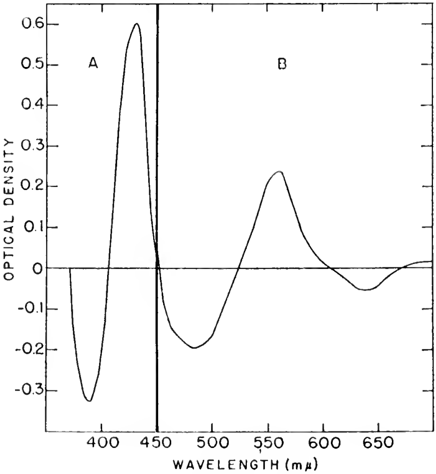

FIG. 2. Difference spectrum of *R. rubrum* RHP at pH7. (Reduced-minus-oxidized) in *A*, 0·66 × 10^{-2} μmole/ml and in *B*, 2·7 × 10^{-2} μmoles/ml in 0·07 M phosphate were used. (Reproduced by permission of the *Journal of Biological Chemistry*.)

moves approximately 10 mμ toward the blue. Because of its extreme photo-instability at physiological pH, the complex absorption spectrum must be measured in very weak light.

5. The α-band of the reduced form is diffuse and appears to consist of at least two components. These are easily seen using a Hartridge reversion spectroscope, but are hardly discernible in the Cary traces, particularly those of the *R. rubrum* compound. The wavelength maxima shown in Fig. 3 are those determined visually, using the spectroscope. The absence of a β-band in the usual position (approx. 520–525 mμ) is another criterion employed in monitoring RHP.

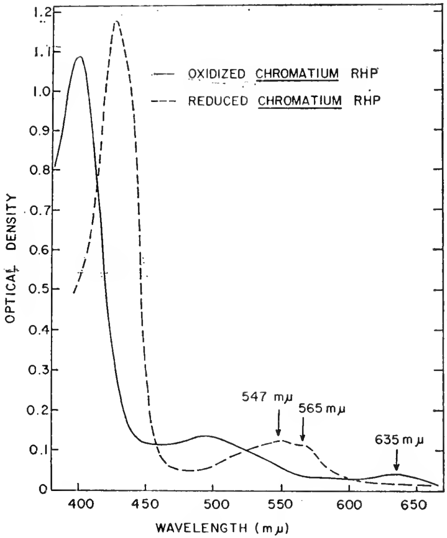

FIG. 3. Spectrum of oxidized and reduced *Chromatium* RHP at pH 6·8.

6. The haemochrome spectrum of RHP found in alkaline medium (Fig. 4) exhibits absorption maxima located at the same positions as those of corresponding cytochrome *c* at physiological pH, but relative intensities of the absorption peaks differ markedly (Bartsch and Kamen, 1958).

In Table 2 we have listed values for a number of extinction coefficients, both for absolute and difference absorption spectra. The preparations measured all had purities in excess of 95%, based on spectroscopic data. They were homogeneous by criteria based on sedimentation and diffusion studies.

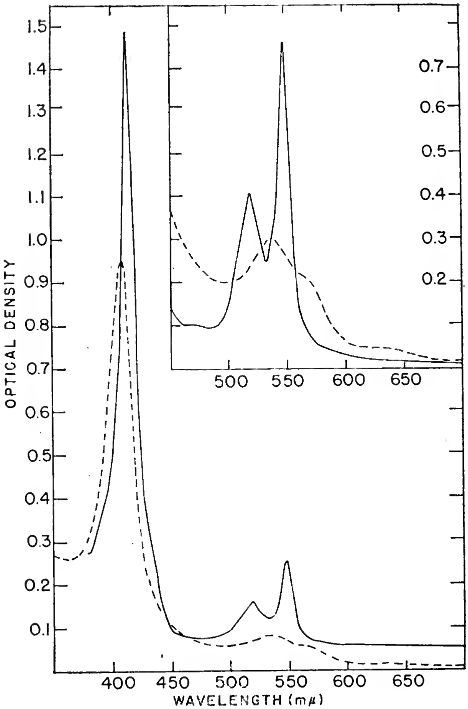

FIG. 4. Spectrum of oxidized (broken line) and reduced (solid line) *R. rubrum* RHP at pH 11·8 (phosphate buffer, 0·1 M). Relative concentrations for main curves and inset curves as in Fig. 1. (Reproduced by permission of the *Journal of Biological Chemistry*.)

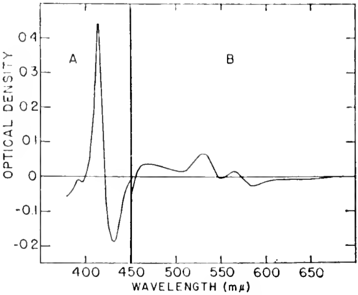

FIG. 5. Difference spectrum of *R. rubrum* RHP-CO compound minus reduced RHP, at pH 4·75. In *A*, 0·27 × 10^{-2} μmole/ml and in *B*, 2·7 × 10^{-2} μmole/ml were used in 0·1 M citrate 2-amino-2-hydroxymethyl-propan-1:3-diol (Tris)-buffer. Gas phase in the experimental cuvette was pure CO.

TABLE 2. EXTINCTION COEFFICIENTS FOR RHP*

(Newton and Kamen, 1956; Bartsch and Kamen, 1958)

State of cytochrome	*R. rubrum*		*Chromatium*	
	Wavelength of absorption peak (mμ)	$E_{\text{sp.}}$‡	Wavelength of absorption peak (mμ)	$E_{\text{sp.}}$‡
Oxidized	275	1·71	280	1·75
	390	6·12	400	5·34
	500	0·83	495	0·68
	640	0·23	635	0·20
Reduced	424	6·73	426	5·83
	550	0·84	547	0·63
			565	0·58
Difference spectrum (reduced–oxidized)	388		398	−3·20
	432		429	4·40
	485		495	−0·40
	550		565	0·15
	640		635	−0·12
Carbon monoxide complex (reduced)	415		418	16·20
	534†		535	0·78
	560†		563	0·66
Difference spectrum (CO, reduced–reduced)	415		418	11·2
	535		535	0·20
	560		567	0·10
$E_{\text{protein}}/E_{\text{soret (reduced)}}$	—	0·25	—	0·3
$E_{\text{soret (reduced)}}/E_{\alpha\text{(reduced)}}$	—	8·01	—	10·0
$E_{\text{protein}}/E_{\text{haematin}}$	—	7·43	—	8·7

* Values in this table corrected in proof; see Horio and Kamen, 1960.

† pH = 4·75.

‡ Specific extinction values are tabulated; to calculate $E_{m\text{M}}$ values, multiply *R. rubrum* values by 26 and *Chromatium* values by 36. These values are provisional, awaiting more accurate dry weight determinations.

As previously shown for physical properties (Table 1), the two preparations differ somewhat in light-absorption properties. A major source of error which contributes to an estimated uncertainty of 10–15% in the values quoted, can be traced to the difficulty in determining dry weight of small amounts of protein. The small differences in location of absorption maxima are to be expected. Similar, and even greater, differences are characteristic of other classes of haemoproteins.

Metal Content

The limited amounts of pure material available have led us to defer determined attempts to establish the content of iron and other metals. We have noted erratic and unreproducible results in the few attempts made with the *Chromatium* preparation, using the customary methods for iron analysis, based on colour development with 1,10-phenanthroline, after acid or alkaline ashing (Sandell, 1944; Drabkin, 1941). The *R. rubrum* preparation has been somewhat more amenable (Bartsch and Kamen, 1958). It yielded acceptably-reproducible iron assays after wet-ashing with nitric acid (Sandell, 1944), but not with alkaline peroxide (Drabkin, 1941). The iron content of *R. rubrum* RHP indicated a minimum molecular weight of 31,000 which was reckoned to be high rather than low owing to incomplete recovery of iron, often met with in assay of standard cytochrome *c* preparations using this method. Inasmuch as the molecular weight determined, either by physical methods or by spectroscopic means using the derivative pyridine haemochrome, was close to 28,000, it was concluded that no iron was present in excess of that accounted for by the haem.

Early studies on the mixed RHP-cytochrome *c* preparation originally obtained in impure and partially denatured form from *Chromatium* (Newton and Kamen, 1956) indicated considerable iron in excess of that which could be attributed to the haem moieties.

Recently, after failure of our preliminary attempts to determine the iron content of the *Chromatium* RHP using wet-ashing and colorimetric procedures, we have begun a collaborative research with Dr. B. L. Vallee, who, with his colleagues, has developed accurate methods for analysis of a wide variety of trace metals in biological systems using dry-ashing and arc or spark emission spectroscopy (Vallee, 1955). We cannot provide definitive data as yet, but tentative results obtained in the first experiments on *Chromatium* RHP show iron contents which are in accord with the haem content.

Since the spectroscopic emission methods can be readily extended to determine all trace metals of possible interest, we plan to investigate the possibility that not only iron but other metals such as copper, manganese, cobalt, nickel, and zinc may be present. It is by no means certain that iron is the only functional metal in these preparations. The close association of RHP and some other haematin compounds with the photosynthetic system raises many questions about a possible photochemistry of these compounds. Such a possibility is difficult to conceive on the basis that iron is the only metal atom present, but would become real should it be demonstrated that metals are present which are photochemically active as bound ions or porphyrin chelates.

Enzyme Properties

Little is known about enzymic activities or cellular functions of RHP. In *R. rubrum* extracts, as well as in *Chromatium* extracts, there are diphosphopyridine-nucleotide (DPN)-linked cytochrome *c* reductases, very active diaphorases, and catalases. The cytochrome *c* reductase of these bacteria can reduce RHP using reduced diphosphopyridine nucleotide (DPNH) as a hydrogen donor (Kamen and Vernon, 1954). *R. rubrum* RHP itself, in the reduced state, spontaneously reduces the corresponding *R. rubrum* cytochrome *c*, as well as mammalian cytochrome *c* (Kamen and Vernon, 1954). This behaviour is reminiscent of that shown by the mammalian microsomal cytochrome b_5 when incubated with cytochrome *c* (Velick and Strittmatter, 1956). The specific DPNH-linked cytochrome b_5 reductase of liver microsomes (Strittmatter and Velick, 1956) fails to catalyse the reduction of RHP with DPNH. The *Chromatium* preparation, in its purified form, has not been tested using *Chromatium* extracts rich in the DPNH-linked cytochrome reductase. Early efforts to establish the enzymic character of *Chromatium* RHP in cytochrome *c* were negative but inconclusive, because the haem complex used was a mixture of the two pigments in an undefined state of purity and at least partly denatured (Newton and Kamen, 1956).

The possibility that RHP might be a peroxidase has been eliminated by the demonstration that all peroxidatic activity in extracts of *R. rubrum* or *Chromatium* concentrated in fractions other than that containing RHP.

Is RHP an Artifact?

We have discussed elsewhere the possibility that RHP is an artifact (Bartsch and Kamen, 1958). Briefly, the reasons for dismissing this possibility are at least as cogent as those for considering other haemoproteins, such as cytochrome *c*, as real constituents of cells rather than artifacts produced by isolation procedures. In one respect only is evidence lacking, and that is the enzymic basis for the existence of this novel haemoprotein.

The reasons for considering RHP as a bona fide haematin component of living cells may be recapitulated here.

1. It can be seen as a spectroscopic entity in living cells (Bartsch and Kamen, 1958).
2. It is obtained by mild extraction procedures in yields equal to or better than those found using more drastic methods involving relative extremes of heat and acidity (Bartsch and Kamen, 1958).
3. It is not formed from other cellular haem components by the extraction and isolation procedures used (Vernon and Kamen, 1954; Bartsch and Kamen, 1958).
4. Difference spectra of actively metabolizing cells, determined under a wide variety of different metabolic conditions, can be correlated with known spectroscopic constants and chemical behaviour of isolated and purified RHP (Chance and Smith, 1955; Olson and Chance, 1958; Smith and Ramirez, 1959).
5. It is one of the most abundant proteins in the bacteria in which it is found. As an example, it is present in *R. rubrum* in amounts as high as 0·6% of all cellular protein. Although this figure is minimal, it is at least as high as that of the bacterial cytochrome *c* content, which is one of the

highest known. The relative amounts of RHP and cytochrome *c* are in accord with those estimated visually in chromatophores after acetone treatment to remove the photoactive pigments.

Reliable methods for accurate estimation of total haem content in *Chromatium* and *R. rubrum* are not available so that it is not possible to say what fraction of the total haematin content is accounted for as RHP, but it is very likely that this fraction is higher than 10%.

CLASSIFICATION OF RHP

Morton (1958) has proposed that RHP be considered the prototype for a new group of cytochromes which is to be labelled 'D'. This group includes the 'new respiratory enzyme' (carbon monoxide-binding pigment) of a number of facultative bacteria described by Chance and co-workers (Smith, 1954; Castor and Chance, 1959), and the RHP-like pigment of the halotolerant nitrate-reducing *coccus* described by Taniguchi *et al.* (1958). There is little question that the anomalous character of RHP, as isolated, requires a revision of the nomenclature accepted at present, but sufficient evidence based on actual isolation of the various pigments, hitherto detected as spectroscopic entities only, is not at hand to strengthen the rather tenuous assumption that all of these haematin compounds are closely identical with RHP chemically.

In the one cytochrome with an RHP character described as occurring in a non-photosynthetic system, that of Taniguchi *et al.* (1958), the preparation shows the characteristic heterogeneous Soret peak in the reduced form, as well as the haematin band in the oxidized form. There is also the proper ratio of intensities between the haematin band, the α- and Soret bands of the reduced form, as well as the same location of absorption maxima. The α-band of the reduced form shows the characteristic heterogeneity exhibited by RHP. However, there is also a marked β-band in the reduced form, located at approximately 521 mμ, which is not characteristic of RHP. It is probable that this preparation contains some cytochrome *c* admixed with the presumed RHP. The hydroxylamine reductase activity of RHP, as obtained from *R. rubrum* or *Chromatium* has not been investigated, so it cannot be said whether RHP is like the haematin compound of the halotolerant *coccus* in this respect. However, it remains to be demonstrated that there is a correlation between this activity and the haematin content of the coccal preparation, inasmuch as the preparation, while electrophoretically homogeneous, has a very high molecular weight, a very low iron content (approximately 0·03%), and shows spectroscopic evidence of heterogeneity. Further purification may resolve these difficulties and confirm the coccal pigment as a haematin compound of the RHP type.

In view of the present uncertainties both with regard to distribution and function, it may be premature to adopt RHP as the basis for a new group of cytochromes. From the long-range view we feel it is probably best to avoid addition of yet another letter group to the three already in existence, especially when it is considered that RHP and its presumed analogues among the non-photosynthetic bacteria may be only structural variants of cytochrome *c*. Whatever terminology is adopted, it should be sufficiently flexible to include not only RHP, which represents a myoglobin-type haematin compound, but also other kinds of cytochrome variants which are possible on the basis of present concepts of haemoprotein structure.

ADDENDUM

Further studies have required some revision of results as originally reported at the *Symposium*. The improved values (Horio and Kamen, 1960) have been incorporated in Tables 1 and 2. It has also been found (Horio and Kamen, 1960) that the minimum molecular weight of the *R. rubrum* preparation is 12,900 (based on iron estimation) and 13,000 (based on estimation of pyridine haemochrome). The molecular weights of *R. rubrum* RHP and of *Chromatium* RHP as determined by physical methods have been found to be 26,000 and 36,000 respectively. The corresponding minimum molecular weights, as indicated by estimation of pyridine haemochrome and of iron content are 12,900 and about 17,000 respectively. Thus there appear to be two haem moieties per mole of RHP in both preparations.

Acknowledgements

Much of the early work on which this report is based was made possible by the financial support of the C. F. Kettering Foundation and by the Linde Air Products Company. Continued support throughout the later period of the research has come from the National Science Foundation and the National Institutes of Health, whose help we gratefully acknowledge. Permission to reproduce Figs. 1, 2, 4 and 5 has been graciously extended by the Editor of the *Journal of Biological Chemistry*.

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DISCUSSION

The Functions of Cytochrome b$_4$ *and of Cytochrome* c544
(Halotolerant Coccus)

EGAMI: I would like to summarize the results on cytochrome b_4 and on a CO-binding, autoxidizable pigment of a halotolerant bacterium, which Kamen considered to be similar to his RHP.

Both cytochromes were discovered in my laboratory (in Nagoya) in a halotolerant micrococcus. Halotolerant and halophilic bacteria, which we like to use for the extraction of bacterial enzymes, have an advantage in that they lyse easily in distilled water or in dilute salt solution. Thus we can easily extract various bacterial enzymes. Cytochrome b_4 was extracted thus in 1953. CO-binding autoxidizable cytochrome *c*544 (halotolerant micrococcus) was also extracted in the same way later.

Cytochrome b_4 transfers electrons to nitrite and hydroxylamine through nitrite reductase and hydroxylamine reductase respectively. Cytochrome b_4 itself is halophilic, e.g. it functions as an electron-transferring enzyme only in relatively concentrated salt solution. Hori and Taniguchi purified 'cytochrome b_4' by DEAE-cellulose chromatography and it was shown that 'cytochrome b_4' consisted of a *c*-type cytochrome (main component), and a *b*-type cytochrome. Even after purification, the former has a double-peak α-band.

CO-binding, autoxidizable cytochrome *c*544, although not yet obtained in homogeneous state, is presumably identical with hydroxylamine reductase. Hydroxylamine reductase activity is enhanced by Mn^{++}. According to Taniguchi, the saturation concentration of NH_2OH for the enzyme increased with increasing concentration of Mn^{++}. It is suggested that Mn^{++}, binding NH_2OH and the enzyme (cytochrome *c*544), participates in the electron transfer between the two substances.

Nomenclature of CO-*binding Pigments*

MORTON: The names 'RHP', cytochrome *o* and cytochrome *d* call for some comment. In a recent review on cytochromes (Morton, *Rev. pure appl. Chem.* **8**, 161, 1958), I attempted to gather together existing information on properties of cytochromes. As pointed out at that time, I did not wish to propose a new nomenclature but adopted the names in common usage. Only in the case of the 'CO-binding pigments' did I consider it was necessary to point to probable relationships between the pigments of different micro-organisms and I adopted the classification 'cytochrome *d*' for this class of compounds. I purposely left out cytochrome a_3 (which combines with CO) since it is so well established in the literature.

I was not aware, of course, that Castor and Chance (*J. biol. Chem.* **234,** 1587, 1959) had also proposed a group name for these compounds (but had used another nomenclature) since their paper had not appeared. The name 'cytochrome *d*' for this class was suggested provisionally since it was apparent that further information about these pigments was required. I now feel that neither of these group names (viz. 'cytochrome *d*' and 'cytochrome *o*') should be further used and that only descriptive classifications such as 'RHP-type pigment of *Chromatium*' or 'CO-binding pigment of *M. pyogenes var. albus*' should be used until the Commission on Enzyme Nomenclature has given a decision on cytochrome nomenclature. This would probably lead to less confusion among biochemists generally.

Cytochrome *o*

By B. Chance (Philadelphia)

Chance: In 1954, Lucile Smith and I discovered a photodissociable carbon monoxide compound in several types of bacteria, the absorption bands of which were similar to haemoglobin. In view of this similarity and of Keilin and Tissières' studies (*Nature, Lond.* **172,** 390, 1953) of haemoglobin in some micro-organisms, we proceeded cautiously to ascribe a function to it. Not long thereafter, Castor, with his improvement of Warburg's photochemical method, showed the compound to be a terminal oxidase (Castor and Chance, *J. biol. Chem.* **217,** 453, 1955) and in 1959 named the oxidase cytochrome *o* (Castor and Chance, *J. biol. Chem.* **234,** 1587, 1959) (Fig. 1).

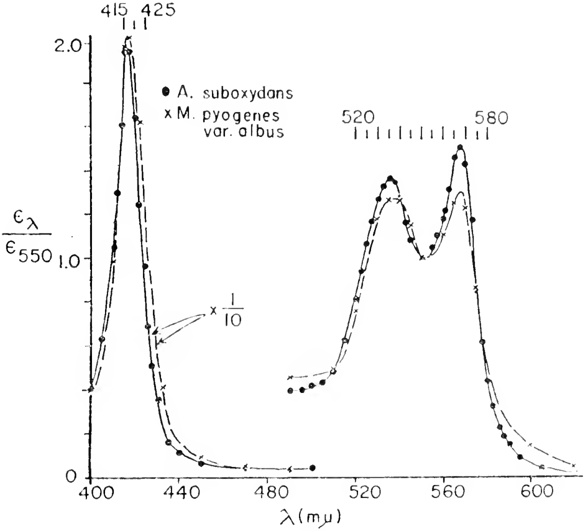

Fig. 1. Photochemical action spectra for cytochrome *o* of *A. suboxydans* and *M. pyogenes* var. *albus* (from Castor and Chance, *J. biol. Chem.* **217,** 453, 1955). Reprinted with permission of the *Journal of Biological Chemistry.*

At about the same time, Kamen and Vernon (*J. biol. Chem.* **211**, 643, 1954) began the study of RHP–CO in purple bacteria. This compound shows, as does cytochrome *o*, a CO-myoglobin type spectrum. Table 1 further compares the properties of the two compounds There is fairly close agreement between the peaks of their absorption bands, but the photochemical data give a larger γ/α ratio for cytochrome *o*. The higher extinction coefficient of RHP–CO may strengthen the possibility that this haem is a polymer, perhaps a dimer. (This has recently been found true for both *R. rubrum* and *Chromatium* RHP (Kamen and Horio, personal communication).)

TABLE 1. COMPARISON OF THE ABSORPTION CHARACTERISTICS OF CYTOCHROME *o*–CO WITH THOSE OF RHP–CO

	α	β	γ	γ/α	$\varepsilon_{\gamma}{}^{\text{mM}}$
Cytochrome *o*–CO	568	536	417	14·7	80–90
RHP–CO	560	534	415	10·4	$\frac{220}{2} = 110$*

* Kamen, personal communication.

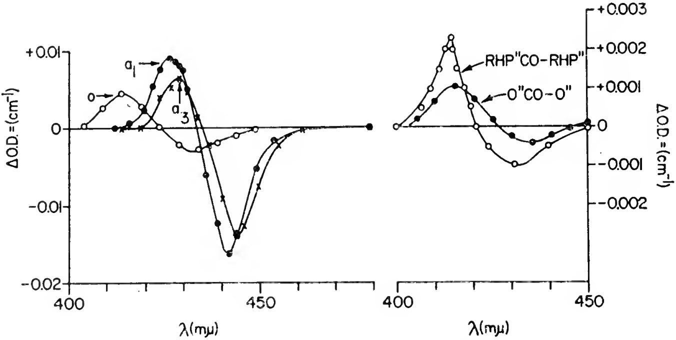

FIG. 2. Photodissociation difference spectra comparing cytochrome *o* with cytochromes a_1 and a_3 (left) and with RHP–CO (replotted from Kamen and Bartsch, this volume, p. 426).

A comparison of the difference spectra of the two compounds is afforded by Fig. 2. On the left, cytochrome *o* is compared with and clearly distingushed from cytochromes a_1 and a_3. On the right, cytochrome *o* is compared with RHP–CO and a considerable similarity is observed.

The speed with which cytochrome *o* is oxidized can at present be determined in the rapid flow apparatus by following the α band of cytochrome of type *c* (Fig. 3), a method that indicates only the minimum value of the reaction velocity. The reaction is slow for *M. pyogenes* var. *albus*, but is somewhat faster for *A. suboxydans* (right).

A comparison of such kinetics with those of RHP in *R. rubrum* is not possible because of the optical artifacts caused by the flow of the spirillum in the observation tube. A similar study with *Chromatium* gives satisfactory results, but the oxidation of the cytochromes is very slow.

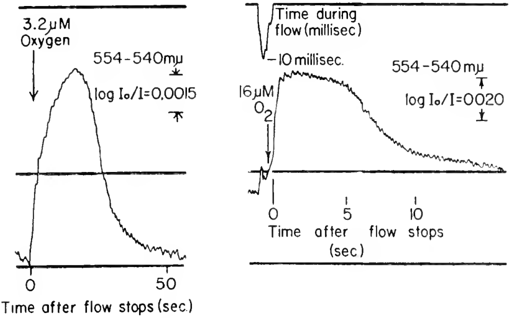

FIG. 3. Rapid flow studies of the speed of oxidation of cytochrome of type *c* by cytochrome *o* in *M. pyogenes* var. *albus* (left) and *A. suboxydans* (right).

On the Oxidase Function of RHP

Smith: In regard to the oxidase function of the RHP of *Rhodospirillum rubrum*, as discussed by Kamen, we have not been able to find any respiratory activity in isolated chromotophores of these organisms, although other fractions of the broken-cell suspensions can respire. Also we cannot find any evidence for the formation of the CO-compound of reduced RHP in anaerobic suspensions of *R. rubrum* that have been grown in the dark with aeration, although they respire very rapidly. Finally, an oxidase function for RHP is very puzzling in *Chromatium*, since it is an obligate anaerobe.

Kamen: We have noted that RHP could hardly function as an oxidase in *Chromatium*. In fact we regard it as likely that the protein in *R. rubrum* and other facultative bacteria may function as an oxidase whereas in *Chromatium* it may retain only an electron-transferring property. Another uncertainty relates to the fact that cyanide inhibits *R. rubrum* respiration whereas there is no spectroscopic evidence of combination between RHP and cyanide. However, this also appears to be the case with *Pseudomonas* oxidase (as reported by Horio *et al.*, this volume, p. 302) where cyanide inhibits without showing visible alterations in the pigment spectrum. As for the experiments on *R. rubrum* grown in the dark, it would be a matter of great difficulty to interpret results of such an approach. We know there are profound changes in haematin enzyme content with such culture conditions and that not only RHP, but also catalase, peroxidase and the various cytochromes fluctuate greatly. At present, there are no compelling reasons for discarding the oxidase hypothesis.

Addendum (*in proof*). Horio, working in Chance's laboratory with Dr. C. P. S. Taylor, has now found (September, 1960) that the CO-action spectrum for oxidase activity in dark-grown aerobic *R. rubrum* is identical with the CO-reduced RHP spectrum.

SPECTROPHOTOMETRIC STUDIES OF CYTOCHROMES COOLED IN LIQUID NITROGEN

By R. W. Estabrook

Johnson Foundation for Medical Physics,
University of Pennsylvania, Philadelphia 4

INTRODUCTION

One of the primary problems associated with the studies of biological oxidations is the recognition and identification of those components participating in the electron transport chain during the oxidation of substrates by oxygen. The classical spectroscopic studies of Keilin (1925) showed the presence of absorption bands characteristic for specific intracellular haemoproteins, which he called cytochromes. These studies, coupled with the considerable numbers of enzymic tests carried out by Keilin and his collaborators, have served as the foundation for our present understanding of cellular respiration. The fact that the various haemoproteins have, in their reduced form, specific absorption bands in the visible spectra has served as the means of identification, thus permitting an investigation into the mode of interaction of cytochromes. An excellent summary of the spectral properties of cytochromes has recently been published by Morton (1958).

One of the more recent developments in the spectroscopic identification of the cytochromes are the observations, first published by Keilin and Hartree in 1949, describing the remarkable effect of lowering the temperature on the sharpening of the associated absorption bands of reduced cytochromes—thus permitting the resolution of overlapping absorption bands. This effect coupled with an intensification resulting from the devitrification of the glycerol media employed by Keilin and Hartree (1949) permitted them to recognize and characterize cytochrome c_1 (Keilin and Hartree, 1955) having *in vitro* properties which suggest its function as a member of the respiratory chain, thus confirming the earlier contention of Yakushiji and Okunuki (1940) concerning the presence of such a pigment as cytochrome c_1. In addition, Keilin and Hartree (1949) recognized the splitting of some of the absorption bands of reduced cytochrome c giving rise to fine structure or what they termed satellite bands.

The use of low temperature spectroscopy is not new. Many varied types of pigments such as porphyrins, nucleotides, chlorophyll and carotenoids have been investigated at low temperature and the appearance of additional band structure has frequently been observed. The organic solvent systems (such as the ether–pentane–alcohol (EPA) mixture) generally employed by chemists in the spectroscopic studies of these pigments are inadequate, however, for work with proteins. Thus the introduction by Keilin and Hartree (1949) of a glycerol–water-mixture as a medium and the resultant five to tenfold intensification of absorption bands obtained with such a mixture, has served as the basis for the identification of pigments in such low amounts that they would not otherwise be discernible.

We at the Johnson Foundation, recognizing the importance of Keilin's and Hartree's (1949) work on the low temperature effect but cognizant of the restriction imposed by simply describing spectra observed in a low dispersion spectroscope, have adapted a wavelength scanning recording spectrophotometer in order to plot automatically the spectra of haemoproteins at low temperature. These studies have confirmed in every way the excellent reports of Keilin and Hartree. Indeed, we can honestly say that we have not been able to add much to the basic knowledge brought forth by them other than to extend the range of material studied.

The present paper will deal with only two facets of the studies carried out at the Johnson Foundation; the first is principally concerned with the satellite band structure of soluble haemoproteins, and the second with the complexities observed in the spectra obtained with particulate materials.

METHODS

Figure 1 presents a schematic description of the instrument employed. This is a more modern version of the basic instrument which has been designed and developed by Chance and his colleagues (Chance, 1951; Yang and Legallis, 1954). A Bausch and Lomb diffraction grating monochromator serves as the source of monochromatic light; the monochromatic light from the diffraction grating is divided into two equal parts by an oscillating mirror and then passes through the cuvettes in the sample holder to an end-on photomultiplier. As with all spectrophotometric studies of turbid material the geometry of the sample to the light detector is most important, i.e. the cuvettes must be placed as close to the face of the photomultiplier as possible so that the greatest solid angle of transmitted and scattered light will be included. For use at low temperatures (Estabrook, 1956), an unsilvered Dewar flask is placed in the position normally localized for the sample. By filling this Dewar with liquid nitrogen and using an appropriate sample holder, one is able to cool samples and thus record the spectra of haemoproteins at the temperature of liquid nitrogen.

Figure 2 shows in greater detail the arrangement of the sample holder and Dewar flask. Two cuvettes are employed, one being essentially a reference, the other the sample to be investigated (Note 1). The two light beams pass alternately through the two cuvettes and the difference in light absorption,

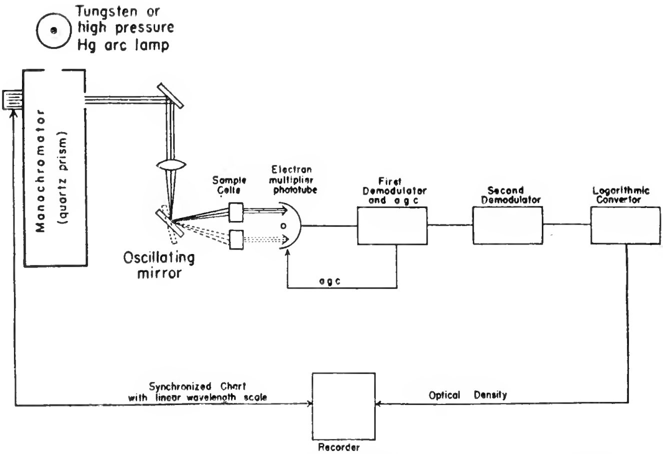
Tungsten or
high pressure
Hg arc lamp
Monochromator
(quartz prism)
Oscillating
mirror
Sample
Cells
Electron
multiplier
phototube
First
Demodulator
and a g c
Second
Demodulator
Logarithmic
Convertor
a g c
Synchronized Chart
with linear wavelength scale
Optical Density
Recorder

FIG. 1. Schematic description of the wavelength scanning recording spectrophotometer developed by Chance (1951).

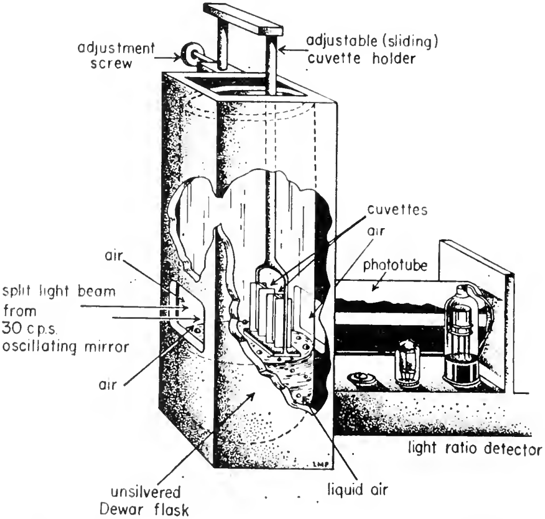
adjustment
screw
adjustable (sliding)
cuvette holder
cuvettes
air
phototube
air
split light beam
from
30 c.p.s.
oscillating mirror
air
light ratio detector
unsilvered
Dewar flask
liquid air

FIG. 2. Detail of the cuvettes and holder used for low temperature spectrophotometry.

after appropriate amplification, is then recorded automatically as a function of wavelength. The sample holder is arranged so that the samples may be raised out of the Dewar, put into liquid nitrogen, or aligned in the light path from the monochromator.

A classic demonstration of the performance of a spectrophotometer is the spectrum which one can obtain with a didymium nitrate solution. In Fig. 3 are plotted two spectra as obtained using the instrument developed by Chance. The spectrum marked AA is that of didymium nitrate at room temperature—a rather complex spectrum. When the sample is cooled in liquid nitrogen and then warmed to devitrify the glycerol mixture and then recooled in liquid nitrogen, one obtains the spectrum BB (Note 2). There is a tenfold difference in the optical depth of the cuvettes employed in the two experiments: that is, the upper curve, BB, should be ten times larger than the curve AA. Of interest here, other than seeing the appearance of additional absorption bands at liquid nitrogen temperature, is the resolution of absorption bands which one can obtain with the instrument we are using. For

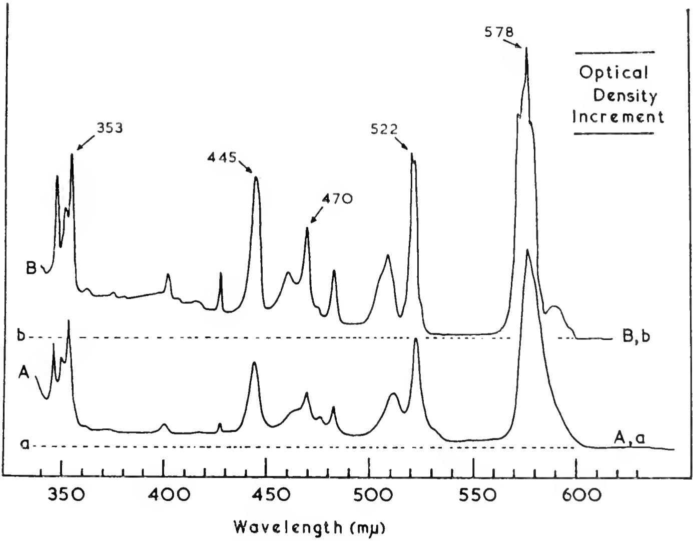

FIG. 3. Spectra of didymium nitrate. A 10% solution of didymium nitrate was mixed with an equal volume of glycerol and the spectrum was recorded at room temperature (A–A) using cuvettes with a 10 mm light path. A sample of the didymium nitrate–glycerol mixture was placed in a cuvette of 1 mm light path and cooled in liquid nitrogen and the spectrum recorded (B–B).

example, at 522 mμ we are able to resolve absorption bands which are approximately 10 Å apart. Another point of interest is the fact that one obtains as great an intensification of the associated absorption bands in the visible region around 570–580 mμ as can be obtained at 350 mμ or 445 mμ. That is, there is a fifteen to twenty fold intensification of all absorption bands throughout the spectrum.

CYTOCHROMES OF THE *c*-TYPE

Cytochrome c *of Heart Muscle*

Figure 4 shows the type of spectrum one obtains (Estabrook, 1956) with cytochrome *c* purified from horse heart (Note 2). The sample is reduced, in this case, by sodium dithionite although identical results are obtained if the cytochrome *c* is reduced enzymically. The spectrum of the same sample of reduced cytochrome *c*, as obtained at liquid nitrogen temperature, has been included. One of the first differences observed is that the α-band of reduced cytochrome *c*, which at room temperature looks relatively symmetrical, splits into three absorption bands (Note 3) which we have termed $c_{\alpha 1}$, $c_{\alpha 2}$, $c_{\alpha 3}$. The β-band shows an even more complex structure and by precise spectral analysis one sees nine absorption bands. In addition one sees an intensification of the light absorption by these α- and β-bands of five to six fold. In contrast, the Soret band does not appear as greatly intensified. In addition one sees no clear splitting of the Soret band, indeed all that is

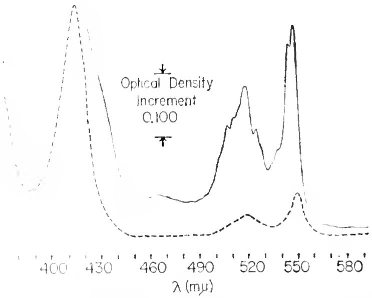

FIG. 4. A comparison of the spectral properties of heart muscle cytochrome *c* at room temperature and at −190 C. Purified cytochrome *c* was diluted in 0·1 M phosphate buffer, pH 7·4, a few crystals of $Na_2S_2O_4$ were added, and then an equal volume of glycerol. The reference cuvette contained a mixture of an equal volume of glycerol and 0·1 M phosphate buffer. The sample and reference cells were cooled in liquid nitrogen, warmed to induce devitrification of the glycerol mixtures, and recooled in liquid nitrogen (Condition II). Optical path of the cuvettes was 1 mm.

observed is the appearance of a shoulder at about 430 mμ indicative of another possible absorption band. A point of interest is the appearance of what have now been termed (Estabrook and Sacktor, 1958) beta prime (β') bands, a triple band structure at about 470 mμ. Although of low extinction

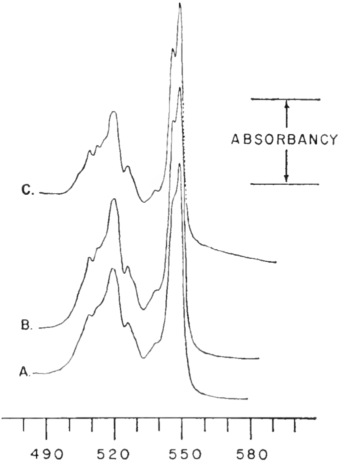

Fig. 5. The effect of glycerol on the appearance of the $c_{\alpha 2}$ absorption band of reduced cytochrome *c*. Horse heart muscle cytochrome *c* was diluted in 0·2 M phosphate buffer, pH 7·4 and reduced by the addition of $Na_2S_2O_4$. Various volumes of a mixture of 75% glycerol, 25% 0·2 M phosphate buffer, pH 7·4 (v/v) were added to portions of the reduced cytochrome *c* solution to give varying concentrations of glycerol. Curve *A* represents no glycerol, curve *B* was with 14% glycerol, and curve *C* contained 71% glycerol. Optical depth of cuvettes, 3 mm.

these three bands appear to be present in all the purified cytochrome haemoproteins that have been studied so far.

Of interest is the nature of the splitting of the α- and β-bands observed in samples cooled in liquid nitrogen. One immediately asks does this imply heterogeneity of the sample, thus indicating the presence of more than one haemoprotein? If the split of the α- and β-bands as observed is not due to contamination by more than one haemoprotein, what is the significance of this split. How may the fine band structure, as observed when samples are cooled in liquid nitrogen, be modified and what factors influence these bands? It may also be asked whether the changes seen in the spectra of modified samples of cytochrome *c* can be related to the biological activity of the haemoproteins, to the protein structure itself, or to the spatial arrangement of the constituent parts of the molecule?

Effect of Glycerol

When samples of reduced cytochrome *c* are diluted in various concentrations of glycerol and then cooled in liquid nitrogen, it is observed that glycerol is required in order to resolve clearly the $c_{\alpha1}$ and $c_{\alpha2}$ absorption bands. This is illustrated in Fig. 5 where three representative spectra are presented. When the concentration of the glycerol is less than 5% (v/v) the absorption band attributed to $c_{\alpha2}$ appears merely as a shoulder on $c_{\alpha1}$. In addition, some of the β-bands as well as the $c_{\alpha3}$-band are less distinct. This failure to obtain maximal resolution in the absence of glycerol may be due to the type of crystal structure formed when the sample is cooled to -190°C, or it may represent a hitherto unrecognized reaction of reduced cytochrome *c* with glycerol forming a derivative with distinctive absorption bands discernible only at liquid nitrogen temperatures. In the subsequent discussion most of the measurements have been carried out in the presence of about 50% (v/v) of glycerol in order to measure the maximal effect of low temperature on the absorption bands of reduced haemoproteins.

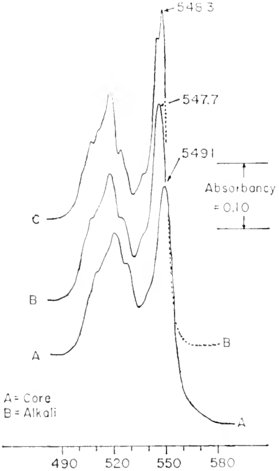

FIG. 6. A comparison of the spectral properties of reduced cytochrome *c* of heart muscle, the alkaline haemochrome of cytochrome *c*, and the peptide core of cytochrome *c*. Curve *A*—Samples of peptide core of cytochrome *c* were dissolved in 0·1 M phosphate buffer of pH 7·4 and a few crystals of $Na_2S_2O_4$ added. This was then mixed with an equal volume of glycerol. Curve *B*—Samples of horse heart cytochrome *c* were diluted with 1 N NaOH, permitted to incubate 10 min at room temperature, reduced with $Na_2S_2O_4$, and then mixed with an equal volume of glycerol. Curve *C*—Samples of heart muscle cytochrome *c* were treated as described in Fig. 4. Optical depth of cuvette was 1 mm.

Modified Cytochrome c *of Heart Muscle*

The extreme in modification of α- and β-band splitting is observed when one determines the spectrum of the peptide core of cytochrome *c* (Ehrenberg and Theorell, 1955). This is shown in Fig. 6 (Note 4). Included with the spectrum of digested and reduced cytochrome *c* is a spectrum of alkali-modified cytochrome *c* as well as that of biologically-active heart muscle cytochrome *c*. One sees with the peptide core a single α-band, broad and symmetrical, with indications of a $c_{\alpha 3}$-band at 536 mμ. The presence of a complex β-band is apparent but the many small bands generally observed with the undigested cytochrome *c* are not detectable. Alkali-treated cytochrome *c* has also lost much of the fine band structure. Of interest is the 1·5 mμ difference in absorption band maxima between the two modified samples. The spectrum of the peptide core of cytochrome *c* resembles most closely the alkaline haemochrome of reduced cytochrome c_1 (see below).

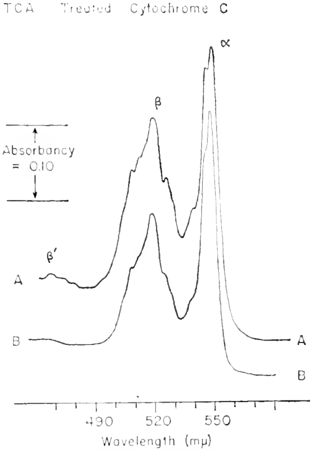

Fig. 7. Spectra of trichloroacetic acid-modified cytochrome *c*. Samples of horse heart cytochrome *c*, obtained from Dr. Margoliash, were treated as described in Fig. 4. Curve *A* represents fraction I_a while Curve *B* represents fraction II_c as obtained from the ion exchange resin IRC 50 during the purification of cytochrome *c*.

Figure 7 shows the extremes of a series of samples which we were fortunate to obtain from Dr. Margoliash. These represent the first (A) and the last (B) of a series of six fractions of cytochrome *c* obtained from IRC 50 resin (Margoliash, 1954a) during the purification of horse heart cytochrome *c* prepared by the conventional Keilin and Hartree (1945) method. Margoliash

(1954b) has termed these type I and type II cytochrome *c*. One sees that the first material to be eluted (Type I_a) has very well defined $c_{\alpha1}$- and $c_{\alpha2}$-bands. As subsequent samples are eluted, the degree of resolution of these two bands gradually diminishes until we see in the last sample to be eluted from the column (Type II_c) that there is merely a small shoulder indicative of the presence of $c_{\alpha2}$. Margoliash, Frohwirt and Wiener (1959) have characterized this last fraction as a trichloroacetic acid (TCA)-denatured cytochrome *c*, and have investigated in detail the chemical and enzymic activities of these fractions, showing that the TCA-modified cytochrome *c* has lost a large part of its enzymic activity. Thus another means of modifying the split of the absorption bands observed at low temperature is by acid denaturation of the cytochrome *c*.

Cytochrome c *from Other Sources*

One further means of obtaining a difference in the fine band structure of cytochrome *c* is to prepare the pigment from other sources. We have purified the pigments and investigated the spectra of cytochrome *c* from wheat germ, flight muscle sarcosomes of the house fly, rat livers, and yeast. All of the samples assayed, except that from yeast (Note 5), showed essentially

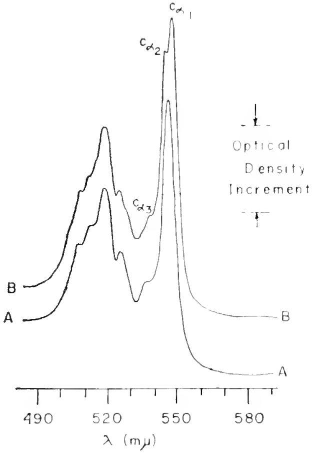

FIG. 8. A comparison of the spectral properties of reduced cytochrome *c* prepared from heart muscle and from yeast. Samples of cytochrome *c* diluted in 0·1 M phosphate buffer, pH 7·4 were reduced with $Na_2S_2O_4$ and diluted with an equal volume of glycerol. Curve *A* represents the spectrum obtained with yeast cytochrome *c* while Curve *B* is that obtained using heart muscle cytochrome *c*. Optical depth equals 1 mm. Condition II.

the same type of fine band structure of the α- and β-bands as heart muscle cytochrome *c*, with some modifications in the location of the maxima.

Figure 8 presents a comparison of the spectra obtained when samples of heart muscle cytochrome *c* and yeast cytochrome *c* are analysed. The spectrum of the yeast cytochrome *c* looks similar to that obtained with TCA-denatured or with alkali-denatured heart muscle cytochrome *c*. It should be noted, however, that the locations of the absorption bands of the denatured material and of the yeast cytochrome *c* are different.

Other Types of Cytochrome c

Some other interesting variations on pigments of the type of cytochrome *c* are shown by results obtained with cytochrome c_4 of *Azotobacter vinelandii.*

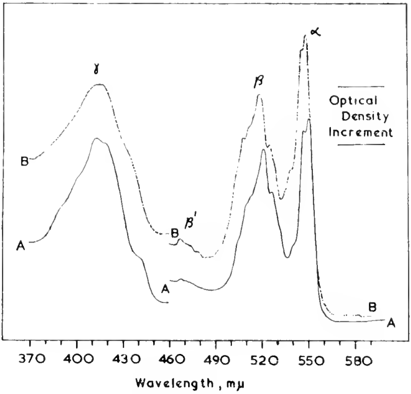

FIG. 9. A comparison of the spectral properties of reduced cytochrome *c* prepared from heart muscle and cytochrome c_4 of *Azotobacter vinelandii.* Curve *A* represents the spectrum obtained with cytochrome c_4 while curve *B* is that obtained with heart muscle cytochrome *c*. Optical depth equals 1 mm. Condition II.

One sees in Fig. 9 the spectrum of reduced cytochrome c_4 (curve A) compared to that of the heart muscle cytochrome *c*. Of interest is the fact that even though the fine band structure of the α- and β-bands are apparently identical in both samples, the location of the maxima of the absorption bands of reduced cytochrome c_4 from bacteria are shifted about 1·5–2 mμ to the red. It was difficult to convince ourselves of the validity of this observation, but repeated experiments have proven that this is not an artifact of the machine or the method of carrying out the experiments, but truly a reproducible difference between the two pigments. The multiplicity of Soret bands observed with cytochrome c_4 prepared from *Azotobacter* is in contrast to the results shown for cytochrome *c* from heart muscle where very slight changes were seen when the sample was cooled in liquid nitrogen. The bacterial cytochrome c_4 shows much greater detail in the Soret region, at least five absorption bands being apparent.

Another series of *c*-type pigments which have been investigated are the pigments cytochrome c_1 of heart muscle and c_5 of *Azotobacter*. These are shown in Fig. 10. The spectrum of heart muscle cytochrome *c* is added as a dotted curve to orient one in locating wavelengths. One of the first observations is that cytochromes c_5 and c_1 do not show a distinct splitting of the alpha absorption bands. There is however, a pronounced splitting of the β bands. Although these two pigments have been derived from entirely different organisms, i.e. heart muscle and *Azotobacter vinelandii*, they have identical spectral characteristics in every way thus far measurable. It should be mentioned, however, that there are differences in biological activity as well as chemical properties between the two pigments. Cytochrome c_1 functions (Keilin and Hartree, 1955; Estabrook, 1958) between cytochrome *b*

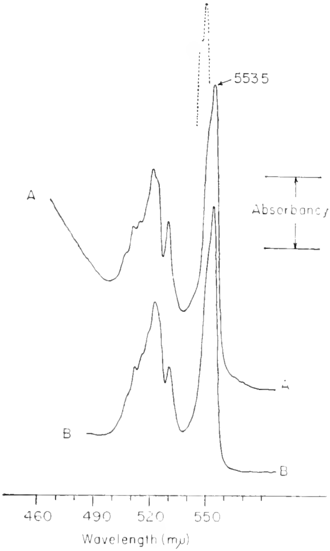

FIG. 10. A comparison of the spectra of cytochrome c_1 and c_5. Curve *A* represents the spectrum of cytochrome c_1 obtained as a difference spectrum of cytochrome c_1 reduced by ascorbic acid minus the oxidized pigments of a heart muscle DPNH-cytochrome *c* reductase. Curve *B* is the spectrum of cytochrome c_5 of *Azotobacter vinelandii* obtained with $Na_2S_2O_4$ as reducing agent. Condition II.

and *c* in mammalian tissues and has an oxidation-reduction potential of about +0·145 V, while cytochrome c_5 of *Azotobacter* has an oxidation-reduction potential of about +0·300 V (Tissières and Burris, 1956; Tissières, 1956). In addition, cytochrome c_5 is enzymatically active in particles of *Azotobacter*, whereas it is not active in the heart muscle system.

CYTOCHROMES OF THE *b*-TYPE

Cytochrome b_4

A pigment which we have been most fortunate to have available (Note 7) is cytochrome b_4 from the halotolerant bacteria. As shown in Fig. 11, the α and β absorption bands of reduced cytochrome b_4 show the most remarkable splitting of any pigment yet observed. The two α absorption bands which are observed at low temperature have their maxima about 6 mμ apart. Another

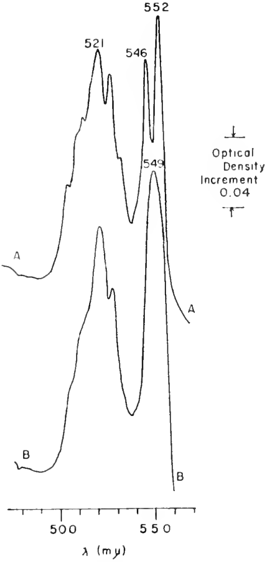

FIG. 11. Low temperature spectra of purified cytochrome b_4 from halotolerant bacteria. The upper curve represents the spectrum observed when purified cytochrome b_4 is diluted in 0·1 M phosphate buffer, pH 7·4 and reduced with $Na_2S_2O_4$. The sample was then mixed with an equal volume of glycerol and the spectrum recorded. The spectrum of the alkaline haemochrome of cytochrome b_4 was obtained in a similar manner except that the sample was diluted in 1 N NaOH. Optical depth equals 1 mm. Condition II.

anomalous property of this pigment is the very high extinction of the β bands. In addition it must be mentioned that cytochrome b_4 is really not a b-type cytochrome but has proven to be a c-type cytochrome. This is evidenced by the location at 549 mμ of the alpha band of its reduced alkaline haemochrome. The impure samples of cytochrome b_4 first reported by Egami, Itahashi, Sato and Mori (1953) have since been shown to be contaminated by another haemoprotein with a typical b-type absorption spectrum.

Cytochromes b$_1$, b$_2$ *and* b$_5$

Spectral studies have been carried out on cytochrome b_1 of anaerobic yeast, cytochrome b_2 (yeast lactic dehydrogenase) and cytochrome b_5 of liver microsomes. All three pigments show essentially the same type of absorption spectrum at low temperature (Chance, Klingenberg and Boeri, 1956; Lindenmayer and Estabrook, 1958). As shown in Fig. 12 one observes a split of the α-band of reduced cytochromes b_1 and b_2; it should be noted that in contradistinction to the fine band structure observed with the α-band of reduced cytochrome c of heart muscle, the band of lower extinction

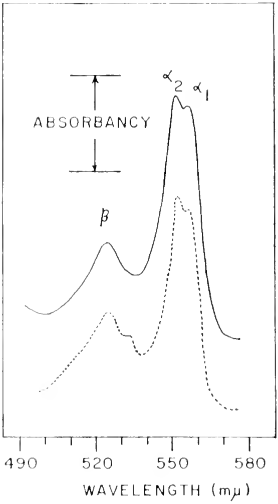

FIG. 12. The low temperature absorption spectra of cytochromes b_1 and b_2. Solid line: cytochrome b_1 in intact cells of anaerobically grown yeast, stationary phase of growth, reduced with $Na_2S_2O_4$. The dashed line represents cytochrome b_2 purified from baker's yeast, reduced with $Na_2S_2O_4$. Optical depth was 1 mm, Condition II.

resides on the red side (long wavelength side) of the major band in these *b*-type pigments, whereas it is on the blue side of the spectrum with cytochrome *c*. Also of interest is the fact that the major absorption band of cytochromes b_1, b_2 and b_5, that is the band of highest extinction, has its absorption maximum at about 553 mμ, approximately the same as that seen for cytochrome c_1 (Fig. 10). The alkaline haemochromes, however, differ between cytochromes of b_2 and b_5 type and the cytochrome c_1 type, the latter having the maximum of the reduced alkaline haemochrome at 549 mμ.

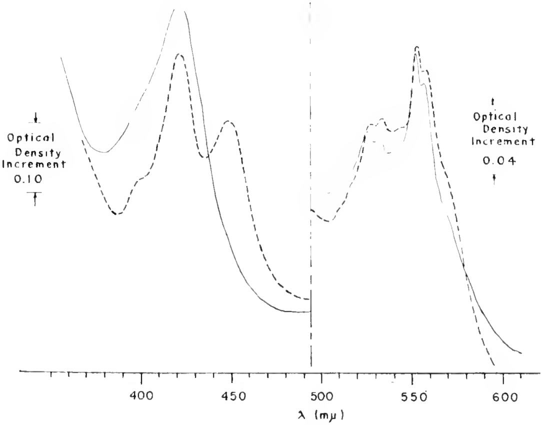

Fig. 13. The pigments of rat liver microsomes. Microsomes were diluted in 0·1 M phosphate buffer, pH 7·4 and then a few crystals of $Na_2S_2O_4$ were added. The sample was then diluted with an equal volume of glycerol. The resulting spectrum (solid line curve) shows the double α-band of cytochrome b_5. A similar sample reduced by $Na_2S_2O_4$ was treated by bubbling with CO to give the dashed line curve. Optical depth was 1 mm. Condition II.

Of principal interest is the type of spectrum one obtains with cytochrome b_5 of liver microsomes. The spectra of the reduced, and CO binding pigments of the rat liver microsomes are presented in Fig. 13. In addition to the split of the α and β bands one sees that the addition of CO causes the appearance of a large absorption band at about 448 mμ, confirming the previous observation of Klingenberg (1958). The split of the α- and β-bands does not appear to be influenced by the presence of CO.

Of interest are two contradictory experiments concerning the interpretation of the split of the α-band of cytochrome b_5 into two distinct absorption bands. In an attempt to resolve the question of whether this represents the contribution of two haematin components (cf. Chaix, Petit, Monier and Zajdela (1958)) or one, the spectral properties of cytochrome b_5 purified by

Dr. Garfinkel (Garfinkel, 1957) from two different sources were investigated. As shown in Fig. 14 there is a difference in the magnitude of the $b_{5\alpha1}$ absorption band when comparing the spectrum of the two samples. These samples of cytochrome b_5 were reduced enzymatically by a specific DPNH-cytochrome b_5 reductase purified by Dr. Garfinkel. The variation observed would indicate that the splitting of the α-band seen with cytochrome b_5 may be due to the presence of more than one pigment, the haemoprotein contributing to the $b_{5\alpha1}$-band having been partially removed during the course of purification.

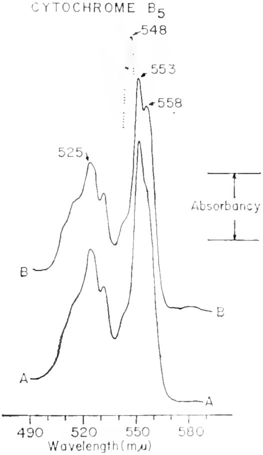

FIG. 14. A comparison of the spectral properties of cytochrome b_5, purified from two different sources. Samples of purified cytochrome b_5 were diluted in 0·1 M phosphate buffer, pH 7·4 and a purified reduced diphosphopyridine nucleotide (DPNH)-cytochrome b_5 reductase and DPNH were added. The sample was then diluted with an equal volume of glycerol and the spectrum recorded. Curve *A* represents cytochrome b_5 purified from rabbit liver microsomes while curve *B* is that obtained using pig liver as the source of microsomes. The dotted curve represents the spectrum of reduced heart muscle cytochrome *c*. Optical depth was 1 mm. Condition II.

If this hypothesis is correct, i.e. that more than one haemoprotein contributes to the spectrum of cytochrome b_5, then a preferential reduction of one or the other pigment might be expected when the system is titrated with DPNH. As shown in Fig. 15 this is not the case. Addition of small amounts of DPNH and then rapid cooling of the sample (see below) shows that both $b_{5\alpha1}$ and $b_{5\alpha2}$ appear together. This indicates either that the two bands observed are associated with a single pigment or that two pigments are being reduced simultaneously to the same extent when a substrate such as DPNH is added.

A point worthy of mention is the possible confusion which may arise because of the similarity of absorption band maxima of reduced cytochrome c_1 and $b_{5\alpha 2}$. This situation reaffirms the contention that spectral studies alone may be misleading and must be coupled with enzymic studies employing various specific inhibitors or substrates in order to distinguish clearly one pigment from another.

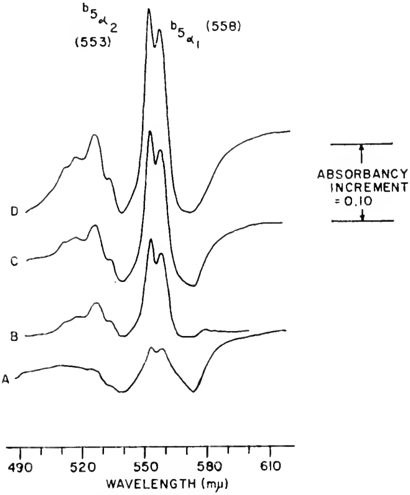

FIG. 15. Spectra showing the titration of cytochrome b_5 of rat liver microsomes by DPNH. Samples of rat liver microsomes were suspended in 0·1 M phosphate buffer, pH 7·4. One portion was removed and placed in the reference cuvette. To the remainder were added small amounts of DPNH. The sample was then cooled by plunging the cuvettes into liquid nitrogen and the difference spectrum recorded. Curve *A* represents the spectrum obtained when DPNH sufficient to reduce 27% of the cytochrome b_5 was added, curve *B* was the spectrum for 54% reduction, curve *C* for 81% reduction while curve *D* was the spectrum obtained with a large excess of DPNH. Optical depth of the cuvette was 3 mm.

PARTICLE-BOUND PIGMENTS

As is apparent from the above, low temperature studies are at present purely descriptive and of principal importance for identification of pigments. The technique has therefore been extended to a study of the respiratory pigment complement of particle-bound haemoproteins from a variety of tissues. Among the first samples to be investigated were the particulate heart muscle preparations; these studies were carried out in collaboration with Dr. Mackler (Estabrook and Mackler, 1957). As shown in Fig. 16 one obtains a resolution of the α absorption bands associated with the cytochromes *b*, *c* and c_1 of heart muscle. Indeed, this method is the most sensitive for determining the presence of cytochrome c_1 and distinguishing cytochrome *c* from cytochrome c_1.

An extension of this type of study is the spectrum shown in Fig. 17 of the respiratory pigments of sarcosomes isolated from the flight muscles of the fly (Estabrook and Sacktor, 1958). These studies, carried out with Dr. Sacktor, show the apparent absence of cytochrome c_1 in this type of material. It is known from other studies, however, that although cytochrome c_1 appears to be absent, a pigment functioning similarly to cytochrome c_1 but of entirely different absorption characteristics is present.

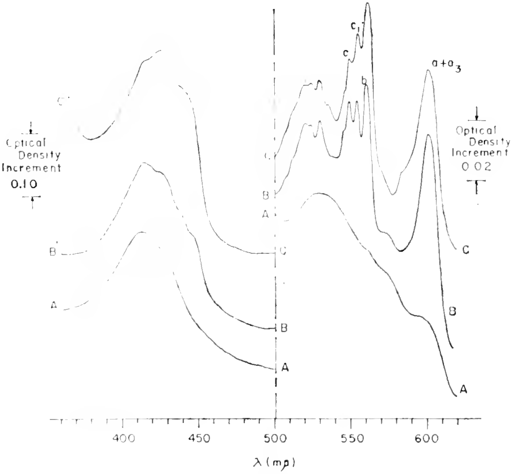

Fig. 16. The low temperature spectra of the reduced haemoproteins of a Keilin and Hartree heart muscle preparation. Samples of heart muscle preparation were diluted with 0·1 M phosphate buffer, pH 7·4, appropriate reagents added, and then mixed with an equal volume of glycerol. Curve *A* represents the spectrum of the oxidized pigments while curve *B* shows those pigments reduced by succinate in the presence of cyanide and curve *C* represents those reduced by $Na_2S_2O_4$. Optical depth was 1 mm. Condition II.

Another type of material studied was the cytochrome complement of wheat germ mitochondria as presented in Fig. 18. This work was carried out with Dr. Stern of the University of Toronto. Again one sees variation in the type of cytochrome spectra obtained. The spectral properties of the cytochromes of plants will be described in greater detail by Dr. Bonner at this symposium. Thus it appears that every new source that we have tried shows a variability in cytochrome pattern. One must admit that it becomes most

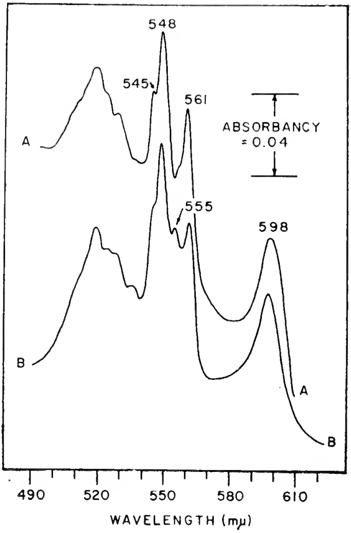

FIG. 17. The low temperature spectra of the reduced pigments of flight muscle sarcosomes. Curve *A* represents the spectrum obtained when the pigments are reduced enzymically. The sample cuvette contained a mixture of 0·4 ml of sarcosome preparation (6·8 mg protein), 0·02 ml of 0·5 M α-glycerol phosphate, and 0·4 ml glycerol. Curve *B* represents the spectrum of those pigments reduced by sodium dithionite. The sample cuvette contained a mixture of 0·4 ml of sarcosome preparation (4·5 mg protein), a few crystals of sodium dithionite, and 0·4 ml glycerol. The reference cuvette contained a mixture of equal volumes of glycerol and buffer. Optical depth of cuvettes was 1 mm; half effective band width, 0·7 mμ; Condition II. Temperature, −190°.

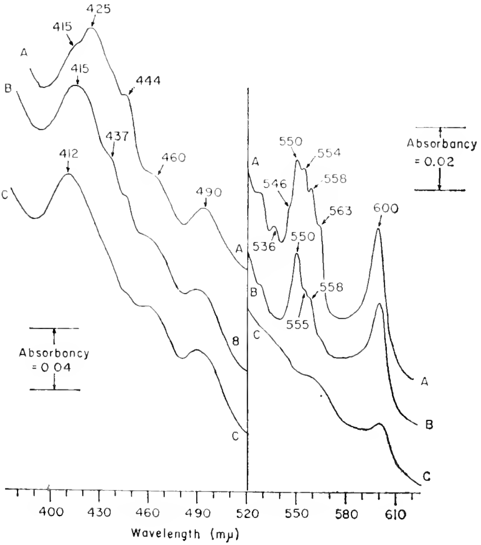

FIG. 18. The low temperature spectra of reduced haemoproteins of wheat germ mitochondria. Samples of mitochondria were treated with various reagents, mixed with an equal amount of glycerol, and cooled in liquid nitrogen. Curve *A* represents those pigments appearing on the addition of $Na_2S_2O_4$; curve *B* is the pigments reduced by succinate in the presence of cyanide; curve *C* represents the absorption spectrum obtained when no reducing agent was added. Optical depth equals 1 mm. Condition II.

intriguing to unravel the nature of the respiratory pigments present in various sources, and indeed certainly makes the hypothesis of a unified cytochrome complement untenable. At the present time the use of low temperature spectroscopy appears to be the most promising approach to unravel the complexity of variable pigment content in different types of materials.

Trapped Steady States

Recently Chance and Spencer (1959), and more recently Chance and Bonner (unpublished), have succeeded in recording the difference spectrum of pigments in various steady states of oxidation. This is shown in Fig. 19, where the difference between the extent of cytochrome reduction in states 4 and 3 of phosphorylating rat liver mitochondria is presented. A clear resolution of the crossover point between cytochromes *b* and c_1 is demonstrated. The combined increase in resolution of absorption bands coupled with a means of rapidly trapping a steady state holds great promise in solving in part the action of the respiratory pigments during oxidative phosphorylation.

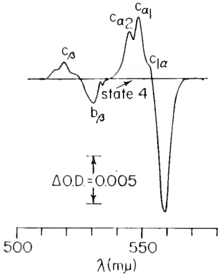

FIG. 19. The low temperature spectrum of the trapped steady states of rat liver mitochondria. Samples of mitochondria, diluted in isotonic buffer, were treated with substrate (state 4). One portion was treated with ADP to oxidize the pigments to state 3. The samples were then rapidly cooled in liquid nitrogen and the resulting difference in light absorption plotted as a function of wavelength.

DISCUSSION

The cooling of samples of reduced haemoproteins, in particular the cytochromes, causes a sharpening of the associated absorption bands and a splitting of some of these bands. The observed narrowing of the bands is caused by restricting the energy loss from the excited vibrational states of the absorbing molecule to the solvent molecules. The lowering of temperature therefore accomplishes in part a change comparable to the sharpening of absorption bands seen when going from the liquid to the vapour state with organic molecules such as benzene derivatives.

The origin of the splitting of the absorption bands is the question of the greatest interest. Since at present interpretation of the absorption bands of haemoproteins is sketchy, any discussion of factors influencing these bands must be largely general, by analogy with simpler systems. The appearance of an absorption band such as the $c_{\alpha 2}$-band of heart muscle cytochrome *c* probably represents the resolution of a vibrational component of the $c_{\alpha 1}$-band which may be supposed to be a single electronic transition. This and other vibrational bands are not apparent at higher temperatures because, as the bands broaden with the increased temperature, they overlap and obscure all fine structure. The question of how various factors influence the resolution, i.e. the narrowing, of an absorption band such as $c_{\alpha 2}$ can only be surmised. Different solvents or solutes that can interact slightly with the haemoprotein molecule may affect the rate of energy loss from the vibrational levels, and hence narrow or broaden the absorption lines corresponding to excitation of the system to these levels. The viscosity of the medium is an important factor. Changes in solvent-bonding may affect the lifetime of the vibrational states. Adsorption of the molecule on crystals of the solvent or ice surfaces (cracks) may, by distorting the molecule, change the degree to which both weak electronic transitions and vibrational transitions are forbidden. The observation that a loss of $c_{\alpha 2}$ accompanies the loss in enzymic activity resulting from a modification of the protein, implies that these bands are profoundly influenced by the associated protein structure surrounding the haemin molecule. The modification of the protein introduces a perturbation resulting in the change of planarity or symmetry of the ring. These changes may be so small, however, that they could not be detected by chemical analysis.

Of interest is the shift in absorption maxima of the α- and β-bands observed with cytochrome c_1 with the retention of an associated fine band structure closely resembling that seen with heart muscle cytochrome *c*. The somewhat lower electronic energy levels observed in this instance, yet the appearance of vibrational transitions the same distance apart, may indicate a small change in a substitutent location resulting in the change of an electronic energy level. This might be accomplished by a change in the arrangement of the associated side chains on the porphyrin ring.

As our understanding of the factors influencing the spectral characteristics of less complicated molecules such as porphyrins and haemins increases, and our knowledge of the chemistry of haemoproteins such as cytochrome *c* increases, we should be able to explain better the changes observed in spectral properties under a variety of conditions. In addition studies which are planned at liquid helium temperatures, giving better resolution of the associated absorption bands, may establish more precise data for accurate spectral analysis. The technique as described, therefore, is best applied to resolve absorption bands of a mixture of pigments in studies of cytochrome interaction in various particulate materials.

SUMMARY

1. The method of recording spectra of samples of haemoproteins cooled to the temperature of liquid nitrogen has been described.

2. The application of this method of study of the fine band structure of heart muscle cytochrome *c*, and the modification of the fine band structure by alteration of the protein, has been described.

3. The spectral properties of reduced cytochromes c, c_1, c_4, c_5 as well as b_1, b_2, b_4 and b_5 are presented.

4. The usefulness of the technique for resolving the complexity of the cytochrome complement is illustrated by studies on heart muscle particles, insect flight muscle sarcosomes, and wheat germ mitochondria.

5. Speculations on the interpretation of modifications of the fine band structure of cytochrome *c* are discussed.

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NOTES

1. Recently Dr. Walter Bonner had modified the cuvette holder permitting a more precise and convenient method for recording difference spectra and for assuring the maintenance of the temperature of the sample at −190°C.

2. These spectra have been termed 'apparent absolute absorption spectra' to distinguish them from 'difference spectra'. The former describes spectra obtained with turbid samples when an induced turbidity, such as obtained with glycerol water mixtures, is used as a reference.

3. The nomenclature introduced to describe these spectra identifies by subscripts the various absorption bands discernible at low temperature—numbering from longer to shorter wavelengths and retaining the conventional distinction of α, β and γ bands and the general classification of *a*-, *b*- or *c*-type of cytochrome.

4. The sample of the peptide core of cytochrome *c* was obtained from Dr. Anders Ehrenberg of the Nobel Institute, Stockholm, Sweden.

5. It is reassuring to report that Dr. Hagihara has examined his samples of crystalline cytochromes *c* from a variety of sources and confirms the observation that the yeast cytochrome *c* differs from cytochrome *c* obtained from mammalian sources in the manner described here.

6. The samples of cytochrome c_4 and c_5 were prepared by Dr. R. Burris and N. Neumann of the University of Wisconsin.

7. Cytochrome b_4 was prepared by Dr. Ryo Sato of Osaka University, Osaka, Japan.

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DISCUSSION

'Trapped' Steady States

By B. CHANCE (Philadelphia)

CHANCE: Various experiments indicate that there is only a small change in the steady state of cytochromes *c*, *b* and *a* in the transition from room temperature to liquid nitrogen temperatures (77°K). Explanations for this are described elsewhere (Chance and Spencer, *Disc. Faraday Soc.* **27**, 200, 1959), and centre about the very similar temperature coefficient of the inter-cytochrome reaction velocities. This method has important applications in the identification of functional cytochromes and in localization of inhibitory interaction sites in the respiratory chain.

Methods

The method is described in Chance and Spencer (*Disc. Faraday Soc.* **27**, 200, 1959), and very briefly consists of modifications of Keilin's and Estabrook's procedures suggested by Bonner and Yocum, namely the omission of glycerol. Modified cuvettes for 1 and 3 mm paths are used. Instead of absolute spectra, difference spectra are used. The steady-state oxidized reference material is washed and starved where possible. It is strongly oxygenated to ensure an adequate supply of oxygen in the filling and freezing interval. The steady-state reduced material is similarly oxygenated and is treated with substrate just prior to pipetting into the cuvette. The cuvette may be pre-chilled so that freezing occurs very nearly on contact with the cooled surfaces. The difference spectrum is then plotted in the split-beam recorder (see Chance, *Methods in Enzymol.* **4**, 273, 1957).

Identification of Respiratory Enzymes

It was pointed out in the discussion of Chaix's paper that the distinction between respiratory and accessory pigments (for definition see Chance, this volume, p. 476), could be simply made by observation of their steady-state reduction. Trapping of this steady state at liquid nitrogen temperatures has the great advantage of combining the precise identification of cytochrome type afforded by the sharpening and splitting of the bands at low temperatures with the test for respiratory function. An example of this technique is afforded by Fig. 3 of Chance (this volume, p. 607) which illustrates a frozen steady state for succinate-treated ox heart mitochondria. In addition to the reduction of cytochromes *a* and *c* to values characteristic of active electron transfer, we find the steady-state reduction of cytochrome c_1 to be readily observed for the first time in this material and, in addition, can calculate its extent of reduction as a percentage of the fully reduced material. This value is very close to that of cytochrome *c*.

Inhibitory Interaction Sites

The cross-over theorem (see Chance, this volume, p. 607) affords a unique method for the localization of inhibitory interaction sites in the respiratory chain. The changes in steady state of cytochromes *b* and *c* may be small and difficult to measure, and in any case, cytochrome c_1 cannot be discerned at room temperatures.

The application of trapped steady states to the study of cross-over behaviour is illustrated by several figures given in the oral presentation, one of which is included in the text of this volume (Estabrook (Fig. 19), this volume, p. 454). We can demonstrate the state 2–4 and 3–4 transitions in the presence and absence of azide. The participation of cytochrome c_1 in these transitions is clearly shown and a cytochrome b–c_1 crossover is demonstrated (see Chance, this volume, p. 607).

The technique has also been applied to cross-over phenomena in intact cells and the increased reduction of cytochrome *b* and *c* characteristic of the inhibited phase of glucose metabolism can readily be observed, although these changes are too small to allow a resolution of cytochrome c_1.

One of the greatest limitations of the low-temperature technique, namely that it affords only identification and gives no indication of function, appears to be overcome with the trapped steady-state method. It is probable that most of the functional and identification test for cytochromes will be done at low temperatures in the future.

Low Temperature Absorption Spectra of Cytochromes in Relation to Structure

Possible Differences in the Prosthetic Groups of c*-type Cytochromes*

MORTON: With reference to the differences in the low temperature absorption spectra noted by Estabrook, it seems possible that there may be small differences in the prosthetic groups of cytochrome *c* from various sources. Not only does yeast cytochrome *c* differ from heart-muscle cytochrome *c* in absorption spectra both at room temperature and at −190°C, but the prosthetic group of native yeast cytochrome *c* is more readily split by treatment with silver sulphate in acetic acid, as previously noted (Armstrong, Coates and Morton, this publication, p. 386). In one sample of aggregated yeast cytochrome *c*, Armstrong in my laboratory observed that the prosthetic group was split by treatment of the cytochrome with 2 N acetic acid only. Unfortunately, treatment with acetone–HCl was not investigated. Falk, Appleby and Porra (*Soc. exp. Biol. Sympos.* **13**, 73, 1959) have reported the isolation from effective soy bean root nodules, by simple extraction with ethyl acetate–acetic acid, of a haem similar to haem *c*.

It appears unlikely that the prosthetic group of yeast cytochrome *c* differs from that of heart-muscle cytochrome *c*, yet these observations suggest that there may be only one thio-ether link to the polypeptide chain in yeast cytochrome *c*, or possibly true ether bonds instead of thio-ether bonds. J. Keilin (*Biochem. J.* **64**, 663, 1956) has pointed out a somewhat related position in respect of cytochrome *h*. Is there any information as to the influence of the protein structure itself on the splitting of the prosthetic group of cytochrome *c* by Paul's silver sulphate method?

MARGOLIASH: We have repeatedly observed that Paul's silver sulphate method for the splitting off of the prosthetic group of cytochrome *c* works very readily with native horse-heart cytochrome *c* and that the haem is entirely liberated within about one hour at 60°C. However, when the peptic 'core' of cytochrome *c*, containing only an 11-amino acid peptide is used, the reaction proceeds much more slowly and takes from 18–24 hr to go to completion. This is contrary to what may be expected, since if anything, the presence of the entire intact protein should interfere with and not facilitate an attack on a bond presumably holding the haem in a crevice.

LEMBERG: While it appears to me unlikely that yeast cytochrome *c* contains mesohaem (it could not then combine by thioether linkages with the protein) I should like to make a plea to chemists interested in cytochromes *c* to investigate the compound obtained by the silver sulphate method splitting in acetic acid more carefully. It should e.g. be possible to differentiate between haematoporphyrin, vinyl-α-hydroxyethyl deuteroporphyrin and ethyl-α-hydroxyethyl deuteroporphyrin. Unfortunately we have obtained some evidence that the silver sulphate method may yield artifacts and requires further study to exclude secondary alterations.

Origin of the Fine Structure in the Absorption Spectra of Cytochromes at −190°*C*

ORGEL: According to Platt's (*Radiation Biology* **3**, 101, 1956) theory of porphyrin spectra the lowest excited state of a metal porphyrin with four-fold symmetry is doubly degenerate and it is the splitting of this degeneracy which produces the more complicated spectra of the free porphyrins since the latter have only two-fold symmetry. I wonder if the splitting which Estabrook observes at low temperatures is a similar but smaller splitting of the degeneracy of the excited state caused:

1. by the different side chains;
2. by the unsymmetrical environment of the protein.

One would anticipate that such splittings would be very small and might only be revealed when the absorption bands become very narrow as they do at sufficiently low temperatures. It should be noted that splittings due to different types of asymmetry might either add up or cancel out depending on the exact geometry of the situation.

As suggested by Margoliash, the asymmetry might be envisaged as due to different groups bound to co-ordination positions 5 and 6 of the iron, above and below the plane of the haem.

ESTABROOK: These possibilities certainly exist. One difficulty in interpretation which must be considered is that one does not see a split of the absorption bands of haemoglobin, myoglobin, catalase, or cytochrome *a* at the temperature of liquid nitrogen. The inability to see a split with these compounds may be resolved when we complete adapting the instrument for spectra at the temperature of liquid helium.

WILLIAMS: The resolution of the fine structure of the α- and β-bands of cytochromes on cooling to low temperature is to be contrasted with the absence of resolution in the Soret band region. If we accept that the resolution is due to coupling of vibrational with electronic transitions then there are at least two explanations. The first is that given by Orgel in which the vibrational structure is due to a breakdown of the four-fold symmetry by substituents; the other arises from the different natures of the electronic transitions involved and would occur for a totally symmetrical porphyrin. The position can be understood by reference to the spectrum of benzene which has a long wave-length band with vibrational structure and a shorter wave-length band without vibrational structure. Moffitt (*J. chem. Phys.* **22**, 320, 1954) has shown how the difference in coupling between two electronic states and the vibrational states can arise through the change in symmetry property of the ground as compared with the two excited states. Until it is known that symmetrical porphyrins do not show vibrational structure of the α- and β-bands it will not be safe to look for explanations in terms of the ground state symmetry of the ring.

STUDIES ON MICROSOMAL CYTOCHROMES AND RELATED SUBSTANCES

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IN THIS paper I shall attempt to review the development of our knowledge concerning the microsomal cytochrome of mammalian liver and related compounds, as this story provides a case history of the problems in identification, characterization, nomenclature and elucidation of biological significance of cytochromes which is one of the major concerns of this Symposium.

IDENTIFICATION, LOCALIZATION AND CHARACTERIZATION

The 'Liver-type' Cytochrome Spectrum

The pioneer spectroscopic studies of MacMunn (1884, 1886) established that haemochrome-like compounds are widely distributed in tissues of vertebrate and invertebrate animals, and that the haemochrome complement of different animal cells differs both with type of tissue and with the species. In examinations of what he termed the 'histohaematin' spectra of tissues, MacMunn noted that heart and skeletal muscle from a variety of species showed, particularly in the presence of a reducing agent, a characteristic absorption spectrum which he labelled 'myohaematin'. This myohaematin spectrum was the four-banded spectrum of what is now commonly regarded as the typical 'muscle-type' cytochrome system, first intensively studied by Keilin (1925) with the strong α-bands of cytochromes *a* (and a_3) at about 605 mμ, of cytochrome *b* at about 562–566 mμ and of cytochrome *c* (and c_1) at about 550–553 mμ respectively, and the fused weaker β-bands of cytochromes at 520–530 mμ. MacMunn noted that the 'histohaematin' spectra of many other tissues were similar to that of muscle, but that spectra of some tissues, most notably liver and the analogous green gland of certain invertebrate groups, and the adrenal medulla, were distinctly different. Characteristically, these 'liver-type' spectra showed a feeble band in the 605 mμ region, and the bands of cytochromes *b* and *c* appeared to be replaced by a single broad band, generally with no distinct absorption maximum, which approximately spanned the region normally bounded by the α-bands of cytochromes *b* and *c*.

Although the 'liver-type' cytochrome spectrum was subsequently observed, with some variations, in homogenates or particulate preparations from several mammalian tissues, its significance remained uncertain. The centre of the broad band, whose full development required the presence of a fairly strong reducing agent, such as dithionite, fell in the range of 555–560 mμ, thus coinciding approximately with the absorption maximum of the microbial component known as cytochrome b_1 (Keilin, 1934) or b' (Fujita and Kodama, 1934). Keilin and Hartree (1940) consequently referred to the broad band of the 'liver-type' spectrum as the b_1 band, and Yoshikawa (1951) ascribed the band to a haemochrome-like cytochrome b', but the component or components responsible were not more definitely established. The early microbial studies (Fujita and Kodama, 1934) had indicated that at least some b_1-like bands resulted from a fusion of the α-bands of cytochromes b and c, and the studies by Keilin and Hartree (1949) of low temperature spectra suggested that b_1 bands in some cases reflected presence of cytochromes b and c and a third haematin component absorbing between b and c, possibly cytochrome e, now known as cytochrome c_1. Slater (1949) also pointed out that in some instances absorption between the bands of cytochromes b and c may reflect presence of contaminants such as denatured protein-protohaemochrome, with an α-band at about 558 mμ. Extracts of liver preparations with organic solvents also showed a haemochrome spectrum with maximum at 560 mμ which was considered to be possibly referable to cytochrome b (Kun, 1951).

Localization of a Distinct Microsomal Cytochrome

The nature of the intrinsic cytochrome components responsible for the 'liver-type' haemochrome spectrum was established in the course of studies in our laboratory on the intracellular distribution of cytochrome components and oxidative enzymes in rat liver (Strittmatter and Ball, 1952, 1954). Spectroscopic and spectrophotometric examination of washed cellular particles isolated from homogenates of perfused rat liver by differential centrifugation indicated that a complete 'muscle-type' cytochrome system, with absorption maxima at 605, 562, 551 and 520–530 mμ in the reduced state, was localized in the mitochondria, although the cytochrome components were present in lower concentration than in muscle. The localization of these cytochrome components in the mitochondria was correlated with the concentration in this particulate fraction of cytochrome oxidase and other oxidative enzyme activities involving terminal electron transport to oxygen. The microsome fraction, which showed only low capacities for terminal respiration, possessed no spectroscopically discernible amounts of the typical cytochrome components of the 'muscle-type' cytochrome system, but contained instead as its major intrinsic pigment a cytochrome component with α-band at 557 mμ which was not observable in the mitochondria. The concentration of the microsomal cytochrome was of the order of 6×10^{-10} moles/mg protein in rat liver microsomes, accounting for nearly 50% of the total haem found in these particles, and no other haemochrome component was discernible provided that the liver had been perfused, or the isolated microsomes adequately washed to reduce to a minimum such contaminants as adsorbed haemoglobin or denatured protein haemochromes. The microsomal cytochrome was a firmly bound, intrinsic component and could be removed from its particulate binding only by enzymic digestion or by treatment with lipid solvents or bile salts, whereas a single washing sufficed to remove readily adsorbed contaminants such as haemoglobin (Strittmatter and Ball, 1952; Strittmatter, 1952 and unpublished work). As it was specifically concentrated in the microsome fraction, the microsomal cytochrome was tentatively labelled 'cytochrome *m*', pending its more definitive characterization (Strittmatter, 1952; Strittmatter and Ball, 1954). The haemochrome absorption spectrum of liver was therefore quantitatively interpretable as due to masking of the rather weak 'muscle-type' cytochrome spectrum of the mitochondria by the relatively intense absorption by a greater concentration of the microsomal cytochrome.

Properties of Particulate Microsomal Cytochrome

The properties and possible biological function of the microsomal cytochrome were first examined in isolated rat liver microsomes, with the cytochrome still in particulate form and presumably still in essentially physiologic state (Strittmatter and Ball, 1952, 1954; Strittmatter, 1952). In the oxidized state, the visible range of the spectrum showed only a slight broad hump with maxima at about 530 and 565 mμ and an intense Soret band at 414 mμ. When reduced by addition of dithionite to a suspension of microsomes, the typical haemochrome spectrum of the cytochrome appeared, with a sharp α-band at 557 mμ, a β-band at 527 mμ and the Soret band shifted to 423 mμ. Neither carbon monoxide nor cyanide (10^{-2} M) at neutral pH altered the oxidized or reduced spectra appreciably. The prosthetic group of the microsomal cytochrome was identified as iron protoporphyrin from spectroscopic studies on the haem extracted from the microsomes and from the spectrum of the pyridine haemochrome formed with intact microsomes. The apparent standard potential of the microsomal cytochrome in its particulate complex was roughly estimated to be about $-0{\cdot}12$ V at pH 7 from reduction titrations in the presence of oxidation-reduction indicators.

As a microsomal component, the cytochrome was reduced rapidly and essentially completely by reduced di- and tri-phosphopyridine nucleotides (DPNH and TPNH), suggesting that it may serve as acceptor of electrons from reduced pyridine nucleotide-cytochrome reductases concentrated in the microsomes. The cytochrome was also readily reduced by cysteine and partially reduced by ascorbate, but was not appreciably reduced by succinate or *p*-phenylene diamine. Following reduction by DPNH or dithionite, the cytochrome of microsomal suspensions could be reoxidized by presence of air, or, more rapidly, by added cytochrome *c* or ferricyanide; the microsomal cytochrome might therefore provide a link in a DPNH-cytochrome *c* reductase system. As the reoxidation by air was not inhibited by cyanide (10^{-2} M) or azide, it appeared that the microsomal cytochrome was not reoxidized via cytochrome oxidase, but might be autoxidizable. The sum of the properties of the microsomal cytochrome suggested that it should be classified in the *b*-group of cytochromes.

Studies with Isolated Microsomal Cytochromes

The nature and relations of the microsomal cytochrome were confirmed and extended in studies with isolated microsomal cytochrome by Strittmatter and Velick (1956a, b) and by Velick and Strittmatter (1956), who first obtained the cytochrome in highly purified state from rabbit liver microsomes, using a procedure involving separation from microsomes by treatment with pancreatic lipase and purification by ammonium sulphate fractionation. The purified cytochrome, with a molecular weight of about 17,000, possessed as prosthetic group one iron protoporphyrin unit per molecule and contained no appreciable non-haemin iron or flavin (Strittmatter and Velick, 1956a). In its absorption spectra the isolated cytochrome agreed well with the particulate form, showing the Soret band at 413 mμ, the oxidized state and peaks at 423, 526 and 556 mμ after reduction with cysteine or other reducing agents. With the purified substance it was possible also to obtain accurate values for the absorbancy indices of the absorption peaks and to observe new broad absorption bands at 355–370 mμ in the oxidized state and at 320–340 mμ in the reduced state.

Oxidation-reduction titrations and equilibrium measurements indicated that the isolated cytochrome behaved as a single, univalent electron acceptor and donor, and from the oxidation-reduction equilibria, the standard potential was calculated to be +0·02 V at pH 7 (Velick and Strittmatter, 1956). This potential value is more than 0·1 V higher than the values estimated for the cytochrome in particulate suspension from either rat or rabbit liver (Yoshikawa, 1951; Strittmatter and Ball, 1952), but the significance of the difference is uncertain at present. The significance of observed apparent standard potentials, particularly those for particle-bound components, are necessarily subject to uncertainty. If it is a real difference, the shift in the apparent standard potential of such a reversibly reduced substance on liberation from its particulate complex might reflect a differential interaction of the oxidized and reduced cytochrome with some components of the microsomal complex. This would be analogous to the shift of apparent standard potential of diphosphopyridine nucleotide (DPN) in the alcohol dehydrogenase reaction which occurs in the presence of stoichiometric amounts of the enzyme, as a result of differential complex formation of the oxidized and reduced form of the nucleotide with the enzyme protein (Theorell and Bonnichsen, 1951; Hayes and Velick, 1954). Alternatively the cytochrome may have been altered during its isolation, perhaps in the incompletely defined enzymic liberation from its particulate complex, although the retention of specific enzymic activities and other properties provides no evidence of such alteration.

The isolated microsomal cytochrome was readily reduced by such reducing agents as dithionite and high concentrations of cysteine. Unlike the particulate form, the purified cytochrome was not directly reduced by DPNH or TPNH, and reduction by DPNH or TPNH required the presence of cytochrome reductases, which have been obtained in soluble form from liver microsomes (Strittmatter and Velick, 1956a, b). A flavin adenine dinucleotide (FAD)-linked reductase specific for both DPNH and microsomal cytochrome, but essentially inactive with TPNH as electron donor or cytochrome *c* as acceptor, was highly purified from calf liver microsomes (Strittmatter and Velick, 1957), and a reductase of similar specificities has been obtained from pig liver particles (Mahler, Raw, Molinari and doAmaral, 1958). On the other hand, the microsomal cytochrome was not reduced by typical DPNH-cytochrome *c* reductase preparations from heart muscle (Strittmatter and Velick, 1956b). The reduced form of isolated microsomal cytochrome was very rapidly oxidized by cytochrome *c*, with a direct cytochrome-to-cytochrome electron transfer, by ferricyanide, and by various dyes of appropriate potential; it was also oxidized, less rapidly, by oxygen (Strittmatter and Velick, 1956a). The highly active DPNH-microsomal cytochrome reductase and the microsomal cytochrome therefore form an efficient microsomal system for electron transport from DPNH to cytochrome *c* and this pathway, which is antimycin A-insensitive, can account for at least one-half of the total DPNH-cytochrome *c* reductase capacity observed with isolated microsomes and added cytochrome *c* (Strittmatter and Velick, 1956b).

In addition to the isolation from rabbit liver microsomes, the microsomal cytochrome has been purified from liver microsomes of pig (Garfinkel, 1957; Krisch and Staudinger, 1958), rat (Strittmatter, unpublished work) and calf (Strittmatter and Velick, 1957), by somewhat similar procedures, and, possibly, from pig liver particles (Raw, Molinari, doAmaral and Mahler, 1958). In so far as the preparations have been compared, the purified cytochromes from the various sources appear to be essentially the same protein, with properties referable to a particular type of group *b* cytochrome. In current usage, the term 'cytochrome b_5' is widely used in referring to the microsomal cytochrome of mammalian liver, and to a number of apparently similar pigments in other biological materials.

DISTRIBUTION OF MICROSOMAL CYTOCHROME AND SIMILAR HAEMOCHROMES

There have been observations in many biological materials and derived preparations of haemochrome-like materials variously described as identical with or similar to microsomal cytochrome, cytochrome *m* or cytochrome b_5, but in many cases the identity of the haemochrome-like materials and their possible relationship to the well-characterized microsomal cytochrome are uncertain by reason of insufficiently rigorous or specific tests of identity. Many of these substances are defined solely or largely on the basis of the absorption spectra of an unpurified preparation, particularly by the appearance of an absorption band in the 555–560 mμ region under fairly strong reducing conditions, or by the demonstration of protoporphyrin haemochrome with such preparations. In many instances these observations appear to represent non-specific absorption, since protohaem may be combined with various non-specific or denatured proteins and with other possible nitrogenous components or contaminants of tissue preparations to yield haemochromes with reduced absorption spectra almost identical with that of the microsomal cytochrome, and adsorbed haemoglobin or denatured protein haemochromes are common contaminants of tissue preparations unless care is exercised to exclude or remove them (cf. Slater, 1949; Strittmatter and Ball, 1952; Strittmatter, 1952; Klingenberg, 1958). Moreover, as the early studies of 'b_1-like' bands already indicated, an apparently homogeneous absorption band may result from a group of absorbing substances (not necessarily including one absorbing maximally at the observed absorption maximum) and caution must be exercised even in interpretation of the absorption spectrum of an optically clear extract or 'solution', particularly one which shows uncharacteristic asymmetries (cf. Fujita and Kodama, 1934; Slater, 1949; Strittmatter, 1952; Strittmatter and Ball, 1952, 1954; Hülsman, Elliott and Rudney, 1958). On the other hand, of course, minor differences of properties between different preparations or different purified substances need not exclude the essential identity of the active substances concerned.

Distribution in Mammalian Tissues

Haemochrome materials with an absorption maximum in the region 555–560 mμ have now been reported in preparations from a number of mammalian tissues in addition to liver, including adrenal medulla (MacMunn, 1886; Huszak, 1942; Ryan and Engel, 1957; Spiro and Ball, 1958), mammary gland (Bailie and Morton, 1955, 1958), intestinal mucosa (Bailie and Morton, 1955), ovary (MacMunn, 1886; Yoshikawa, 1951), pancreas (Palade and Siekevitz, 1956), and kidney (Yoshikawa, 1951), but the identity of the absorbing materials in many cases is not clearly established. As to intracellular distribution, studies on localization of well-defined microsomal cytochrome-like material in cell fractions indicate that it is specifically concentrated and firmly bound in the microsome fraction, suggesting that in the intact cell the cytochrome is an intrinsic component of the endoplasmic reticulum or related membranous structures which give rise to the microsome fraction by the usual fractionation procedures. The possible presence of microsomal cytochrome-like substances in other cell compartments is not excluded, but direct and compelling evidence is lacking. There is indirect evidence (Spiro and Ball, 1958; Bailie and Morton, 1955, 1958; Spiro, 1959) that the cytochrome may appear in certain cytoplasmic 'granules', which in at least some cases appear to be derived from the endoplasmic reticulum, being formed by accumulation of newly synthesized material within the vacuoles of the endoplasmic reticulum (cf. Schneider and Hogeboom, 1956; Siekevitz and Palade, 1958). In addition, Raw and Mahler (1959) reported that pig liver mitochondrial preparations contained cytochrome b_5-like material in appreciable concentrations, although the unambiguous designation of this material as an intrinsic mitochondrial cytochrome b_5 was not possible. Previous studies with rat liver had indicated that the mitochondrial fraction contains either no discernible cytochrome b_5 or only a small amount attributable to microsomal contamination (Strittmatter, 1952; Strittmatter and Ball, 1952, 1954; Chance and Williams, 1955).

Bailie and Morton (1955) have reported that bovine mammary gland microsomes in the presence of dithionite show a strong 557 mμ band of cytochrome b_5 but no other detectable haemochrome bands, while the mitochondria of this tissue showed a 'muscle-type' cytochrome spectrum with no evidence of the microsomal cytochrome.

The predominant haemochrome-like band of the adrenal medulla was variously reported in early studies to be at 559 mμ by Huszak (1942) or 561 mμ by Tsou (1951) and was considered by them to represent cytochrome *b*. Spiro and Ball (1958, 1961) have recently reported that the medullary haemochrome material responsible for this absorption band is apparently concentrated in a 'microsomal fraction' and in the 'epinephrine-rich granules', which probably represent distinct entities and are partially separable from a 'mitochondrial fraction' in which cytochromes *b*, *c*, *a* and a_3 appear to be concentrated. The apparent localization, absorption spectrum and ready reducibility of the predominant medullary haemochrome material by DPNH, cysteine and ascorbate suggest a possible relation to the liver microsomal cytochrome, but the problem awaits a more rigorous characterization and establishment of the unitary nature of the medullary pigment. The presence of a microsomal cytochrome-like substance in the adrenal is indicated anew by the recent isolation of a 'cytochrome b_5' from a 'microsomal fraction' of whole pig adrenals by Krisch and Staudinger (1958). The properties of this preparation in general closely resemble those of the isolated liver microsomal cytochrome, although the question of possible inhomogeneity posed by incomplete reduction of the substance with DPNH and by apparent slight spectral asymmetry is not resolved as yet.

Observations in Invertebrates

While there have been many observations in invertebrate tissues of haemochromes absorbing maximally in the range 555–560 mμ, the most explicit demonstration of a microsomal cytochrome-like component in invertebrates has come from studies of cytochrome components in the *Cecropia* silkworm. Many tissues of the larval form, most notably the midgut wall, showed on reduction a broad indivisible absorption band over the range 551–562 mμ, with maximum at about the centre, which was ascribed to a cytochrome x by Sanborn and Williams (1950). In further studies with larval midgut homogenates, Pappenheimer and Williams (1954) concluded from the absorption spectra and such properties as cyanide-insensitive autoxidizability that this *Cecropia* pigment was a b-type cytochrome and called the material 'cytochrome b_5'. They considered it to be closely related to microsomal cytochrome, but also tentatively identified the pigment with cytochrome e (now termed c_1). The insect cytochrome b_5 has not been purified and its precise relationship with the microsomal cytochrome is not established, but the analogy between the two pigments has recently been extended by observations on intracellular distribution: in both the larval midgut mucosa and the wing epithelium of the developing adult *Cecropia*, the cytochrome b_5 was localized in the microsome fraction while cytochromes b, c, a and a_3 were concentrated in the mitochondria (Shappirio and Williams, 1957).

Plants

An apparently analogous haemoprotein in certain plant tissues is cytochrome b_3, first described by Hill and Scarisbrick (1951). In studies of a variety of non-photosynthetic plant tissues, Martin and Morton (1955, 1957) subsequently localized cytochrome b_3 in microsomal fractions. Similar to the mammalian microsomal cytochrome, cytochrome b_3 shows absorption bands in the reduced state at about 558–560 mμ, 525–529 mμ and 425 mμ, is apparently autoxidizable and does not combine with carbon monoxide.

Micro-organisms

Other than having the general characteristics of a b-type cytochrome, cytochrome b_1 of various bacteria shows no particular relationship to the microsomal cytochrome, but preparations of cytochrome b_2[1] from yeast resemble microsomal cytochrome closely in certain haemoprotein properties, including the position of the haemochrome absorption bands, the reduction of the haem moiety by specific enzyme-linked flavin, and its rapid reoxidation by cytochrome c or ferricyanide. However, the nature and definition of the cytochrome b_2 molecule is in question, since, as crystallized by Appleby and Morton (1954), the molecule contained one unit each of riboflavin phosphate and haem and possessed lactate dehydrogenase activity, while a crystalline preparation of Yamashita *et al.* (1957) was devoid of intrinsic flavin or lactate dehydrogenase activity.

POSSIBLE BIOLOGICAL SIGNIFICANCE

The firm association of the microsomal cytochrome in significant and rather constant amounts with specific metabolically-active intracellular structures in the liver and possibly in other tissues bespeaks for it specific biological roles, probably catalytic in nature, but it is not yet possible to define with assurance the particular biological significance of this cytochrome. However, it might be profitable to review again in the light of present information the several possible roles which we considered in earlier studies to merit special consideration (Strittmatter, 1952; Strittmatter and Ball, 1952, 1954).

Storage Form or Precursor

The microsomal cytochrome might represent a storage form of haemin or a precursor in the formation of other cytochromes or haemin components, but there is no direct evidence that it serves in such relatively inert capacities. Such a role was early suggested for cytochrome b_1 in certain micro-organisms (Keilin, 1933), and was apparently supported by observations that the typical 'muscle-type' cytochrome spectrum of aerobically grown resting yeast cultures is altered during anaerobic growth or in a phase of exponential aerobic growth, with replacement of the cytochrome *b* and *c* bands by a b_1 band at about 555 mμ (Ephrussi and Slonimski, 1950; Chaix and Heyman-Blachet, 1957). However, low temperature studies suggest that the b_1 band represents a fusion of bands (Chaix and Heyman-Blachet, 1957). It is likely that essentially all intracellular haem is maintained in bound forms, since addition of low concentrations of free haematin to cell-free preparations markedly inhibits oxidative activities (Keilin and Hartree, 1947). Protohaem might therefore be stored in bound forms with nitrogenous components, most probably proteins, to form haemochromes that possess absorption spectra very similar to that of the microsomal cytochrome, and in the absence of more specific identification such bound forms might in some preparations be mistaken for the cytochrome.

Active Role in Electron Transfer

The specific and highly active oxidation-reduction properties of the microsomal cytochrome suggest for it a more active role in biological oxidation-reduction reactions. Liver microsomes possess a high capacity for oxidation of DPNH and TPNH; indeed, the DPNH-cytochrome *c* reductase activity of liver is concentrated most markedly in this fraction (Hogeboom, 1949; Strittmatter and Ball, 1954). As noted earlier, the microsomal cytochrome and its reductases comprise an efficient antimycin A-insensitive system that could account for a quantitatively important flow of electrons from DPNH (or TPNH) or other donors. However, the question arises as to whether this system functions biologically, and if so, what substances serve as electron acceptors physiologically.

Terminal Electron Transport

The microsomal cytochrome might serve as a link in terminal electron transport to oxygen either directly or via mediators.

The autoxidizability of microsomal cytochrome may suggest a role as a terminal oxidase, but the low rate of autoxidation militates against any major quantitative significance of such a role. The turnover number of purified rabbit liver microsomal cytochrome with oxygen as electron acceptor is of the order of 1/min, in contrast with the very rapid rate of turnover with cytochrome *c* as acceptor (Strittmatter, unpublished work). A limiting value for the rate of autoxidation physiologically is suggested by the report that the turnover numbers for aerobic reoxidation of microsomal cytochrome in intact liver microsomes in the presence and absence of CN^- are 1·2 and 3·6/min respectively (Chance and Williams, 1954). Thus the DPNH oxidase activity of liver microsomes is very low compared to the DPNH cytochrome *c* reductase capacity of these particles or the DPNH oxidase activity of the mitochondria (Strittmatter and Ball, 1954; Hogeboom, 1949; Hogeboom, Claude and Hotchkiss, 1946). Nevertheless, a terminal oxidase role has been postulated for cytochrome b_5 in systems with low rates of oxygen consumption, such as the cyanide-insensitive respiration of insects (Pappenheimer and Williams, 1954) and the ascorbate-dependent, cyanide-insensitive DPNH oxidase activity of a preparation from adrenal 'microsomes' (Kersten, Kersten and Staudinger, 1958).

Alternatively, reduced microsomal cytochrome is known to be oxidized rapidly *in vitro* by a variety of reversibly reducible substances such as cytochrome *c*, ferric salts and a number of dyes, and analogous electron acceptors *in vivo* might serve as mediators of terminal respiration, with electrons being transported further to oxygen either non-enzymically, if the mediator is autoxidizable, or enzymically. Model oxidase systems may be constructed by addition of mediator systems to microsomal cytochrome, but there is no direct evidence for such a system *in vivo*. Thus, while addition of cytochrome *c* and isolated mitochondrial preparations to microsomal cytochrome permits rapid electron transport from the microsomal cytochrome to oxygen *in vitro* (Strittmatter, unpublished work), it is not known whether significant terminal electron transport from microsomal cytochrome via cytochrome *c* and mitochondrial cytochrome oxidase could occur in the intact cells. In particular, the availability of soluble cytochrome *c* and the accessibility of extramitochondrial electron donors such as soluble cytochrome *c* to the terminal respiratory chain of intact mitochondria *in vivo* are questionable (cf. Chance and Williams, 1955; Wainio and Cooperstein, 1956; Slater, 1958). If a microsomal cytochrome-like substance were localized within the mitochondrial structure, it might more readily be involved significantly in terminal respiration.

While, therefore, microsomal cytochrome might play a role in terminal respiration of cells with low rates of oxygen consumption and may contribute to the usually minor cyanide-insensitive respiration, there is as yet no compelling evidence that this substance is responsible for a significant portion of terminal respiration in actively respiring cells.

'Reductive' Synthetic Reactions

Another possibility which merits consideration is that microsomal cytochrome primarily funnels electrons available from DPNH (or TPNH) to acceptors in synthetic processes that are reductive in character, such as the reductive synthesis of fatty acids or steroids, or which involve elements of both reduction and oxidation, such as hydroxylation reactions (requiring both DPNH (or TPNH) and molecular oxygen). The rather low oxidation-reduction potential of microsomal cytochrome would make it a suitable mediator for transfer of electrons in such processes requiring reductive capacity. The localization of the cytochrome in microsomes would be in keeping with such a role, as the microsomes, or the endoplasmic reticulum from which they are derived, are recognized as the site of or are required for a variety of synthetic processes, including protein biosynthesis (Littlefield, Keller, Gross and Zamecnik, 1955), cholesterol synthesis in liver (Bucher and McGarrahan, 1953), and a variety of hydroxylation reactions required for formation and metabolism of steroids, various aromatic compounds, etc., in the liver or adrenal (Ryan and Engel, 1957; Mason, 1957; Kersten, Leonhäuser and Staudinger, 1958). It may therefore be significant that microsomal cytochrome is present in large concentration in the rather abundant endoplasmic reticulum of liver, an organ with a relatively reductive internal environment and a high capacity for many synthetic reactions, and that other tissues in which microsomal cytochrome or apparently similar pigments are reportedly present are also sites of specialized synthetic processes or of transport phenomena which may involve elements of synthesis.

The possible participation of microsomal cytochrome in hydroxylation of steroids by adrenal microsome preparations has recently received considerable attention (see above). A model system of chelated iron salts, ascorbic acid and oxygen can produce hydroxylation reactions (Udenfriend, Clark, Axelrod and Brodie, 1954), and it has been suggested that the haemin of microsomal cytochrome might similarly provide the site at which electrons from DPNH or TPNH and molecular oxygen might be combined to produce the grouping required for biological hydroxylation reactions. However, there is at present no direct evidence for participation of microsomal cytochrome in hydroxylation or other synthetic processes.

Clearly, the question of the biological functions of microsomal cytochrome and other apparently related cytochrome b_5-like substances is still unanswered, but the lively current interest in this problem gives hope that further insight into the biological significance of these compounds may be obtained in the near future.

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DISCUSSION

On the Nature of Cytoplasmic Pigments of Liver Cells

By B. Chance (Philadelphia)

Chance: There has been much speculation on the possible function of the cytoplasmic pigment, cytochrome b_5, in liver cells, and the desirability of direct experimental data is clearly indicated. We have attempted to determine whether cytoplasmic cytochrome b_5 is oxidized and reduced simultaneously with the mitochondrial cytochromes.

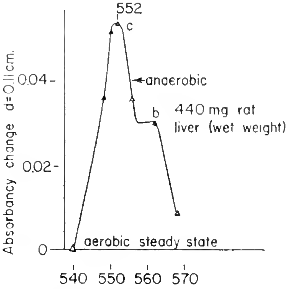

Fig. 1. Steady-state oxidized minus reduced difference spectrum for a rat liver slice. (Expt. 708a).

Liver slices—Studies on liver slices can be carried out by a simple modification of the double-beam spectrophotometer (Chance, in *Methods in Enzymology*, **4**, p. 273. Academic Press, Inc., New York, 1957). Thin slices of perfused liver are floated in Ringer's solution in either an oxygen or a nitrogen atmosphere. Figure 1 records the aerobic-anaerobic difference spectra of such material. There is a peak attributable to cytochromes c and c_1 at 552 mμ and a shoulder caused by cytochrome b absorption at ~562 mμ. There is not, however, any indication that an appreciable portion of cytochrome b_5 (557 mμ) changes its oxidation-reduction level in synchronism with the cytochromes of the respiratory chain.

Other, much earlier work clearly shows what we now term the cytochrome b_5 component to be reduced in liver cells. MacMunn (*Phil. Trans. Roy. Soc. London* **177**, 267, 1886) and Keilin and Hartree (*Proc. roy. Soc. B* **129**, 277, 1940) record reduced bands of this component in liver. Thus, cytochrome b_5 is accessible to reducing substances, presumably cytoplasmic reduced diphosphopyridine nucleotide (DPNH), but not to the mitochondrial oxidase, in accord with studies of isolated mitochondria (Chance and Williams, *J. biol. Chem.* **217**, 395, 1955).

Isolated liver cells—If, instead of the tissue, we use a liver cell suspension (work in collaboration with W. D. Rutter), we find in the low temperature 'absolute' spectra of Fig. 2, the absorption bands of reduced cytochromes c, c_1, b and $a + a_3$ (dashed trace) but no b_5 band. If dithionite is added (solid trace), the absorption of the b components increases considerably and the bands of cytochrome b_5 can be identified. Thus it appears that some if not all of the b_5 component is oxidized in the liver cell suspension and can be reduced only in the presence of dithionite, in contrast with the results on solid tissues. It is possible that cytoplasmic reducing substances, presumably DPNH, have been oxidized or removed in the preparation procedure.

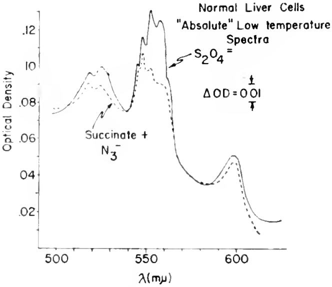

FIG. 2. 'Absolute' low temperature spectra of succinate-reduced (dashed trace) and dithionite-reduced (solid trace) liver cell suspensions. (Expt. 885a).

A further examination of the cytoplasmic pigments of a liver cell suspension at low temperatures is afforded by Fig. 3 in which the oxidized minus succinate-reduced spectrum of the respiratory pigments (solid trace) is compared to that corresponding to the difference between succinate-reduced and DPNH-reduced cells (dashed trace). In the latter, the spectrum of the b components appears, showing the α_1-band of cytochrome b_5. (The slight difference in the maxima of Figs. 2 and 3 can be attributed to differences between an absolute (Fig. 2) and a difference (Fig. 3) spectrum.)

The room-temperature difference spectrum for the same conditions (Fig. 4) does not show the bands described by Williams and myself (Chance and Williams, *J. biol. Chem.* **217**, 395, 1955) at 426 and 557 mμ, but instead shows an apparently different 'cytoplasmic pigment' with absorption bands at 423·5 and 555 mμ. The shoulder on the long wavelength side of the α-band suggests the presence of some cytochrome b_5.

Microspectrophotometry—To localize the pigments in question more accurately, we have constructed a microspectrophotometer capable of recording spectra of respiratory and accessory pigments *in situ* (Chance, Perry, Akerman and Theorell, *Rev. Sci. Instrum.* **30**, 735, 1959). Figure 5 shows a spectrum of an area 1·5 μ in diameter in a single cell teased from a portion of liver tissue after centrifugation of the tissue and rendered anaerobic due to its own respiration (Theorell and Chance, *Exptl. Cell Research* **20**, 43, 1960). Traces 1 represent 'absolute' spectra of various portions of the cell containing the mitochondrial granules; traces 2 represent portions of the

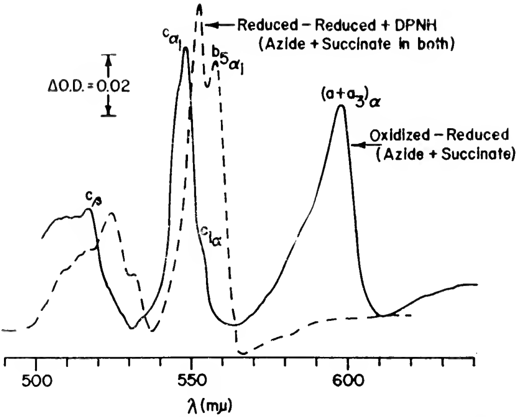

Fig. 3. Oxidized minus succinate-reduced (solid trace) and succinate-reduced minus DPNH-reduced low temperature spectra for liver cell suspensions. (Expt. 976–1.)

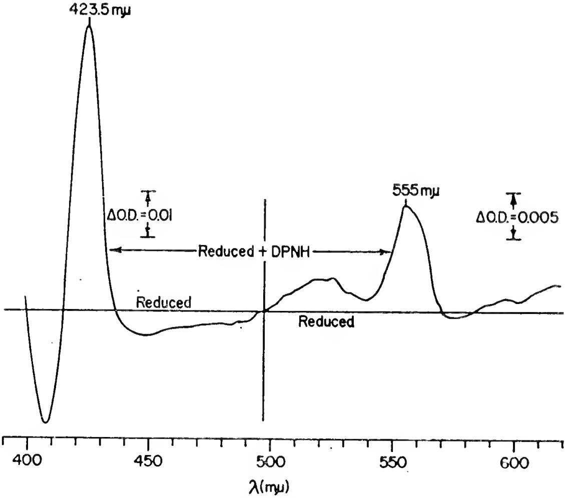

Fig. 4. Room temperature difference spectrum for succinate and DPNH reduced liver cell suspension. (Expt. 979.)

endoplasm where microsomes containing cytochrome b_5 would be expected. The absorption peaks correspond to those of reduced cytochromes a_3, b, and c in the mitochondria and to b_5 or the 'cytoplasmic pigment' in the microsomes. The peak positions are appropriate to the reduced forms of these substances in an 'absolute' rather than a difference spectrum. These data for the single cell teased directly from the tissue indicate a considerable concentration of the reduced 'cytoplasmic pigment' without the addition of DPNH or dithionite, as was necessary for recording absorbency changes of the cells in suspension (Figs. 2 and 3). This result agrees with those of MacMunn and Keilin for liver tissue but clearly distinguishes the location of the cytochromes a_3, b, c and b_5 spectra.

Observations of the cytoplasmic pigment in the transition from aerobiosis to anaerobiosis in cell suspensions support the observations of Fig. 1: no change is observed. However, significant increases of reduction are observed as a function of time after beginning the experiment, which might well be due to variations in the level of cytoplasmic DPNH.

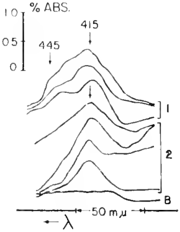

FIG. 5. Microspectrophotometric recording of the spectra of a single, teased and centrifuged, anaerobic liver cell. B, base line; 1, mitochondria; 2, endoplasm. (BT-4.) Reprinted from *Exptl. Cell Research* **20**, 43, 1960.

Nomenclature of accessory and cytoplasmic pigments—These studies and those reported by Chaix (p. 225) and by Bonner (p. 479) lead us to emphasize the use of the term *accessory pigment* (Chance and Williams, *Advanc. Enzymol.* **17**, 65, 1955) in contrast to respiratory pigment. An acceptable preliminary test for the latter is the existence of a steady state of the pigment in respiratory activity as observed at room temperatures or as 'trapped' at liquid nitrogen temperatures. Another test is based on observations of the increasing reduction of the pigment that closely follows the time-course of the termination of respiration as oxygen is exhausted in an aerobic-anaerobic transition. An *accessory pigment* may not change its oxidation-reduction level at all during this transition or may change so slowly that its kinetics violate one of the two theorems mentioned elsewhere in this symposium (see p. 607). A critical test is afforded by a comparison of the kinetics of its oxidation and reduction with the rate of electron transfer through the system. There should be a close relationship for the respiratory pigment and a remote one for the accessory pigment. This does not, however, eliminate the participation of the accessory pigment from slow phases of biological oxidation. If the localization of the accessory pigment is known, then the term may be appropriately modified, as in this case, to cytoplasmic pigment; if the pigment is localized elsewhere, such terms as endochrome, mitochrome, nucleochrome, etc., might ultimately become more useful and more sharply define the general term cytochrome.

Possible Functions of the Cytochromes of the Endoplasmic Reticulum of Animal Cells

DICKENS: My remarks about the paper by Strittmatter (p. 461) concern the possible significance of the reduced di- and triphosphopyridine nucleotide (DPNH and TPNH) cytochrome reductase activity of microsomes. Whereas in the isolated microsome fraction of liver and other tissues cytochrome b_5 readily interacts with DPNH and

TPNH, and the reduced isolated cytochrome is known to be capable of being oxidized efficiently by cytochrome *c*, during the extraction procedure the reactivity towards DPNH and TPNH is lost. It would be of great interest to know precisely on what the TPNH reductase activity depends, for instance, whether a specific pyridine nucleotide reductase such as that found for DPNH is involved, and the nature of its terminal acceptor. Is it possible that, in the intact cell, the fact that the mitochondria lie in the folds of the endoplasmic reticulum, which may therefore be in very close proximity to mitochondria, could facilitate microsomal–mitochondrial interchange? However, Chance's preceding remarks seem to make such an interaction improbable, since there is little change in the oxidation-reduction level of cytochrome b_5 in relation to changes in that of other cytochromes.

On the other hand the microsomes are the seat of an important series of hydroxylation, aromatization and ring-closure reactions which specificially require TPNH, and might therefore represent a main route of TPNH oxidation in microsomes. My colleagues, G. Glock and P. McLean, found for washed liver microsomes a ratio of $DPN^+/DPNH$ of 5/1, suggesting that DPNH is probably capable of oxidation in microsomes, and since DPNH does not appear to be utilized there in the same pathways as those which utilize TPNH, cytochrome b_5 plus its specific cytochrome reductase system would be suitable for DPNH oxidation provided some effective terminal acceptor can be found. There remains, of course, the possibility raised by Chance's comment, that alteration of the haemoprotein occurs during the extraction procedure used.

STRITTMATTER: The possibility of microsomal–mitochondrial interchange to provide a mechanism and possible function for the large pyridine nucleotide oxidizing capacity of the microsomes is indeed attractive, but, as noted in my paper (p. 470), it finds no direct support in the meagre data available. The observation of Chance that microsomal cytochrome in perfused liver slices or cell suspensions is not reduced under mild reducing conditions, in contrast to the mitochondrial cytochrome components, also militates against but does not rule out microsomal–mitochondrial interchange or a respiratory role for cytochrome b_5; reduction of cytochrome b_5 requires a relatively strong reducing agent, such as DPNH, which may be present in the intact cell of solid liver but, as noted by Chance, may have been removed by his procedures for preparing slices or cell suspensions.

The Significance of E'_0 Values of Cytochromes in Relation to Cellular Function

ESTABROOK: Strittmatter and Velick (*J. biol. Chem.* **221**, 265, 1956) have reported on the oxidation-reduction potential of cytochrome b_5. I wonder about the change in potential as determined with the particle bound haemaprotein (−0·120 V) and the purified haemoprotein (+0·02 V), and the significance of this change in any consideration of oxidation-reduction potential and the relevance to a thermodynamic consideration of such potentials in energy transfer.

MORTON: There is a similar type of discrepancy, but in the opposite direction, in the reported E'_0 values for soluble cytochrome *b* of heart-muscle (−0·05 V; Sekuzu and Okunuki, *J. Biochem. Tokyo* **43**, 109, 1956) and for cytochrome *b* in particulate preparations (+0·077 V; Colpa-Boonstra and Holton, *Biochem. J.* **72**, iv, 1959). It would appear that the reactivity of a cytochrome is altered by association with lipoprotein material of cytoplasmic particles.

WAINIO: To demonstrate that the redox potential of an enzyme need not be different when soluble and insoluble, we need only to consider the values obtained for cytochrome *c* oxidase by Ball (+0·29 V) who employed the heart muscle mitochondrial fragment, and by Wainio (+0·285 V), who employed a deoxycholate-solubilized partially-purified preparation of the oxidase.

STRITTMATTER: As was noted in my paper (p. 464), the significance of this apparent change in potential on isolation of the particle-bound cytochrome cannot be assessed with assurance at present. The change, if real, could reflect either the inevitable changes in specific linkages, structural relations and physico-chemical milieu on separation of the cytochrome from its particulate site, or other structural changes resulting from the isolation procedures. The possiblity of either type of change must be considered in assessing the properties of any particulate component removed from its physiological environment, and, on the other hand, the uncertain significance of potential determinations on a particulate component *in situ* must also be kept in mind.

HENDERSON: There is doubt as to the validity of E'_0 values determined on particulate systems in so far as they apply to some molecular species on or within the particle (see also Paul, *The Enzymes*, Academic Press, 1951). This is shown e.g. by the results of Baumberger (Cold Spring Harbor Sympos. Quant. Biol. **7**, 195, 1939) where the degree to which yeast cells were washed and aged caused a variation from +0·12 to +0·31 V in the potential of the medium at which the bands of reduced cytochrome *c* appeared. Apart from any other factors it would seem rather unlikely that the complete thermodynamic reversibility necessary for correct E'_0 evaluation would exist in a complex particulate system and this would be especially the case with the whole cell.

POSTGATE: I think that the absurdity of discussing redox potentials of materials bound at specific sites in cells is illustrated by considering the effect of pH on this quantity. If a bacterium of volume 1 μ^3 has an internal pH of 7, a little arithmetic shows that there are only about 60 hydrogen ions to be expected there. The meaning of pH and hence E_h, at specific sites in the cell is even less meaningful.

THE CYTOCHROMES OF PLANT TISSUES

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INTRODUCTION

Knowledge concerning the cytochrome components and their function in plant tissues has not developed as rapidly as has been the case in animal tissues. This slow development is not entirely due to the fact that, until recently, it was not always considered respectable to work on plant tissues. Visual observation of cytochromes in plant tissues is difficult because of the presence of plastid pigments and the low haem concentration. Plant physiologists themselves, because of their preoccupations with the copper-protein 'oxidases' have been partially responsible for the lack of knowledge of plant cytochromes.

The development of techniques for the extraction and concentration of cytoplasmic inclusions, coupled to the development of rapid and sensitive spectrophotometers, has resulted in considerable progress toward understanding the nature of some of the plant cytochromes. In spite of this progress our present understanding of plant cytochrome components is inadequate and our knowledge concerning the path of electron transport is negligible.

Plant cells possess a rigid cell wall, an immediate barrier to rapid and effective homogenization. This difficulty can be partly overcome by using dark-grown material, the absence of light preventing the large-scale production of lignin. The cytoplasm represents only a modest fraction of the total cell volume, the major portion of a mature plant cell being vacuole. Frequently the vacuolar contents are very acid and on rupture of the cell this acid can produce a considerable and undesirable drop in the pH of the homogenate. This high acidity that is frequently encountered presents an obstacle to the extraction and fractionation of the cytoplasmic constituents of the cell. A completely suitable isotonic extracting medium with low salt content and the necessary buffering capacity remains to be found.

The isolation of the 'mitochondrial fraction' from plant tissues has been more or less stereotyped since the methodology was introduced in 1951 (Millerd, Bonner, Axelrod and Bandurski, 1951; Axelrod, 1955). This method involves homogenization of the tissue in isotonic sucrose together with a rather high concentration of phosphate buffer. In spite of the fact that active, phosphorylating particulate preparations have been reported from many laboratories and with a wide variety of plant tissues, there have been no reports that such particulate preparations exhibited control of respiration by adenosine diphosphate (ADP) concentration. It may be, of course, that in plant tissues there is a more complex control of the respiratory process than that we now understand in animal systems (Chance and Williams, 1956; Chance, 1959).

Many plant tissues exhibit a respiration which is insensitive to the usual ligands, cyanide, azide, and carbon monoxide, a situation analogous to instances recorded in some animal and insect tissues. There are three known types of cyanide-insensitive respiration, viz:

(*a*) The so-called 'ground respiration', easily demonstrated through the prolonged washing of many tissues, e.g. carrot. 'Ground respiration' appears after a marked decrease in respiratory rate during washing.

(*b*) The development of cyanide-insensitive respiration in 'aged' tissue slices; the development of the cyanide-insensitive respiration is accompanied by a marked increase in respiratory rate.

(*c*) Tissues in which the rate of respiration is close to maximum and which normally exhibit cyanide insensitivity. These different situations under which cyanide-insensitive respiration appears in plant tissues add complexities over and above those already enumerated to the understanding of the sequence and function of the cytochromes found in plant tissues.

Cytochromes were first observed in plant tissues by Keilin (1925) who described the reduced spectrum in a wide variety of plant species. Keilin observed that untreated plant tissues showed a two-banded spectrum, a wide diffuse band at 556 mμ and a weaker band at 524 mμ and suggested that this spectrum was due to "modified cytochrome" or cytochrome *b*′. The nature and significance of cytochrome *b*′ remains clouded in obscurity. Following dithionite reduction of the tissue Keilin found that the two-banded spectrum was replaced by a spectrum showing components *a*, *b* and *c*. Curiously, the region of the spectrum between components *b* and *c* appeared shaded and frequently was observed as one wide absorption band.

For fourteen years following Keilin's initial observations the study of cytochromes in plant tissues remained a depressed area. During this time interval, Yakushiji (1935) published on the cytochromes of germinated soy beans in which he described absorption maxima at 640, 603, 560 and 550 mμ. The 640 mμ component was erroneously described as cytochrome a_2. Mann (1938; see also Hill and Hartree, 1953), made an important contribution. He showed that, in general, the greatest amount of haemoprotein, estimated as pyridine haemochrome, was found in the most actively respiring part of the plant tissue, particularly in the non-vacuolated meristematic region.

The year 1939 was a vintage year in the study of plant cytochromes because of the work of three separate groups in Japan, England and America.

Okunuki (1939), continuing investigations initiated in 1937, published three papers devoted to pollen derived from ten different kinds of plants. The cytochrome spectrum of pollen is intense, showing absorption maxima at 605, 561 and 550 mμ. Okunuki did not comment on the difference in maxima of the pollen *b* component (561 mμ) and that of yeast and muscle (564 mμ). Okunuki also studied the effect of carbon monoxide on the respiratory rates of the pollen under investigation and in most instances found a photoreversible inhibition. Remarkably, there were some instances where carbon monoxide had little effect on the respiratory rate, an observation Okunuki interpreted as being due to species variation in carbon monoxide affinity.

Okunuki, in 1939, was successful in preparing and concentrating cell-free pollen extracts which contained a number of dehydrogenases and which were capable of catalysing the rapid oxidation of succinate to fumarate. It is unfortunate that these remarkable contributions of Okunuki have largely been passed over.

Okunuki was primarily interested in the oxidative characteristics of particulate cell-free preparations from pollen. During the same time and using very similar techniques, Hill and Bhagvat (1939) showed that particulate cell-free preparations could be obtained from a variety of flowering plants. Such preparations contained the bulk of the plant cytochromes, observed at that time as components *a*, *b* and *c*, and these cytochromes were observed to undergo reversible oxidation and reduction in plant preparations. Hill and Bhagvat (1939) also observed that their preparations oxidized succinate, and that this oxidation was promoted by the addition of soluble cytochrome *c* and was found to be inhibited by cyanide and azide. The first brief report of Hill and Bhagvat (1939) was elaborated in a later paper (Baghvat and Hill, 1951) in which they stated that components *a*, *b* and *c* could be observed in every tissue studied by them. In each case the cytochromes were associated with cytochrome oxidase and succinic dehydrogenase activities. Bhagvat and Hill (1951) concluded: 'the cytochrome system in plants behaves in the same way as that of yeast and animal tissues, thereby showing the presence of a respiratory mechanism identical with that characteristic of animal tissues'. This very sweeping generalization needs some modification in the light of our present knowledge, much of which has been subsequently contributed by Hill and his colleagues.

Marsh and Goddard (1939) used a different approach for studying the cytochromes of plant tissues. Their approach was to investigate the effects of cyanide, azide, and carbon monoxide on respiratory rates of carrot slices and leaves. By determining the ratio of affinity of the enzymes which mediate the respiration of carrot tissues and young carrot leaves for oxygen and carbon monoxide, these workers deduced that the respiration of these tissues was mediated by cytochrome oxidase. However, mature carrot leaves did not show any appreciable inhibition with azide, cyanide, or carbon monoxide.

From these widely varying approaches to plant respiration substantial evidence was available already in 1939, showing that cytochrome oxidase does mediate the reaction of plant tissues with oxygen and that plants possess a full complement of other cytochromes. In spite of this fact, progress in understanding the cytochromes of plant tissues has continued to be slow for the reasons outlined above.

THE CYTOCHROME COMPONENTS THAT HAVE BEEN DESCRIBED AND NAMED IN PLANT TISSUES

As mentioned above, both Okunuki (1939) and Hill and Bhagvat (1939) and Bhagvat and Hill (1951) described components *a*, *b* and *c*, and prior to this, Keilin had described 'modified cytochrome' or cytochrome b' as well as components *a*, *b* and *c*. Hill and Scarisbrick (1951) described a *b* type cytochrome present in the leaves of a wide variety of higher plants and also extracted from the leaves of *Vicia faba*. The soluble component, called cytochrome b_3, was auto-oxidizable and did not combine with carbon monoxide or cyanide. The presence or absence of cytochrome b_3 has neither been confirmed nor denied, although Martin and Morton (1955, 1957) have described a similar component in the microsomal fractions derived from chlorophyll-free portions of silver beet petioles and from wheat roots. The absorption maxima given by these two groups of investigators are sufficiently different to warrant the view that Martin and Morton were not dealing with the same component described and extracted by Hill and Scarisbrick (see Martin and Morton, 1957).

While studying the cytochrome components in the plastids of etiolated barley leaves, Davenport (1952) observed a *b* cytochrome which was later observed in Chlorella and named cytochrome b_6 by Hill (1954).

There is an additional *b*-type cytochrome component which has been described in the tissues of *Arum maculatum* and called 'cytochrome b_7' (Bendall and Hill, 1956). The term 'cytochrome b_7' has also been used by Bendall (1958), by Hackett and Haas (1958) and by Chance and Hackett (1959). Evidence that cytochrome b_7 differs from previously described *b* cytochromes rests on the visual spectroscopic determination of oxidation-reduction potentials in tissue preparations. This determination is based on the absorption characteristics of preparations that are in equilibrium with various solutions which differ in their oxidation-reduction potentials.

The cytochrome *b*-type components which have been described in plant tissues are summarized in Table 1.

All observations point to the fact that the cytochrome *c* of higher plants is very similar to that of other organisms. Goddard (1944) was the first to extract cytochrome *c* from plant tissues. The cytochrome *c*, extracted from wheat embryos appeared to be identical with the cytochrome *c* extracted from horse heart. More recently, Bonner and Smith (1961) and, independently, Estabrook (this volume, p. 444) have shown that cytochrome *c* from wheat germ has a low temperature absorption spectrum which is identical with that of horse heart cytochrome *c*. Hagihara, Tagawa, Morikawa, Shin and Okunuki (1959) have shown that the crystalline form of wheat germ cytochrome *c* differs from that of cytochrome *c* from other sources.

TABLE 1. CYTOCHROMES *b* THAT HAVE BEEN DESCRIBED IN PLANT TISSUES

Component	Room temperature		−190°C α-max (mμ)	Reference
	α-max (mμ)	β-max (mμ)		
b	564	530	560	Keilin (1925)
				Estabrook (1956)
b_3	559	529	—	Hill and Scarisbrick (1951)
	560	(525)	—	Martin and Morton (1955, 1957)
b_6	562	—	—	Hill (1954)
b_7	560	529	556·5	Bendall and Hill (1956)
			558·5	Chance and Hackett (1959)

It has not been established definitely that plant tissues have a component identical with cytochrome c_1 of animal tissues. Martin and Morton (1957) have used the term cytochrome c_1 to describe an absorption band observed in mitochondria of wheat roots and of silver-beet petioles. Cytochrome *f*, which is in many respects rather similar to cytochrome *c*, was first described and extracted by Hill and Scarisbrick (1951). The extraction procedure was improved by Davenport and Hill (1952). Cytochrome *f* appears to be located in the chloroplasts. Bendall and Hill (1956) have described a cytochrome which is similar to *f* but not identical with it and which they have hesitated to name.

The *a*-components of plant tissues appear to be cytochromes *a* and a_3. Although the evidence is meagre, the few absorption spectra that are available (see Chance and Hackett, 1959; Hackett and Haas, 1958; Yocum and Hackett, 1957; Yocum and Bonner, unpublished work; Hartree, 1957; Martin and Morton, 1957 and Bonner and Smith, 1961) makes it probable that the cytochrome $a + a_3$ band is identical with that found in heart muscle and other sources. There have been many demonstrations of photoreversible carbon monoxide inhibition of a variety of plant tissues. As yet there are no photochemical absorption spectra of carbon monoxide-inhibited plant tissue respiration.

From the foregoing it may be readily appreciated that in the tissues of higher plants the complexities of the cytochrome components are not found in the *c* and *a* components but in the number and diversity of the cytochromes *b*. It is the complexity of the *b* components of plant tissues that gives to them the characteristic visual absorption pattern on reduction, a broad band in the green portion of the spectrum, a band very reminiscent of the absorption spectrum one can observe in well perfused liver slices. The complexity of the number of *b* components in plant tissues immediately raises the question as to their reality. Is it possible that there are as many as five *b* components? Since, on the basis of visual spectroscopy, there have been described since 1951, five different *b* components, is there a possibility that the use of more refined techniques will reveal even more? It is of the utmost importance to answer these questions and also to understand the role that each of the cytochromes *b* plays in electron transport to oxygen and the role, if any, of those components not directly involved in the main transport chain. It is possible that some cytochromes are involved in photosynthesis and in the reactions of photomorphogenesis. All these questions are closely coupled to the structure and function of the various inclusions found in the cytoplasm of plant cells. Therefore, it is of great importance to know the location of each of these different cytochromes *b*, should they turn out to be real. We can begin to answer some of the above questions and try to point a way to the efforts which will have to be invested in an attempt to answer the others.

EXPERIMENTAL

During the past months we have carried out a systematic investigation of the cytochromes of plant tissues with two primary objectives, viz., (*a*) what are the cytochrome *b* components of plant tissues and where are they located in the cell? (*b*) are there species variation in the cytochrome composition of the higher plant tissues? This investigation has been carried out using intact tissue as well as various cell-free preparations derived from them.

The cytochrome components have been delineated through the use of the divided beam spectrophotometer developed by Chance (1957), and adapted for low temperature work by Estabrook (1956). A full discussion of the methodology is given by Estabrook (this volume, p. 436). The reaction cell, illustrated in Fig. 1, was made according to the modification of Yocum and Bonner (unpublished work) who have shown that the glycerol and devitrification procedure of Keilin and Hartree (1949) need not be adhered to. Good spectra can be obtained by freezing in aqueous solution provided a concentrated suspension of particulate matter is used. This procedure lends itself well to the study of frozen steady states. It should be emphasized that the absorption maxima obtained at low temperature in aqueous solution are shifted to slightly longer wave-lengths when compared to those obtained in 50% glycerol at the same temperature.

Since it was impossible to determine the osmotic concentrations of each tissue used in this investigation, one standard procedure for isolation of cytoplasmic inclusions was used; this procedure is outlined in Fig. 2. In cases where the tissues were known to be fairly acid, 0·05 M potassium bicarbonate or 0·01 M Tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer, pH 7·8 were used in place of the phosphate. A more detailed description of the isolation of plant particles is given by Bonner and Smith (1961).

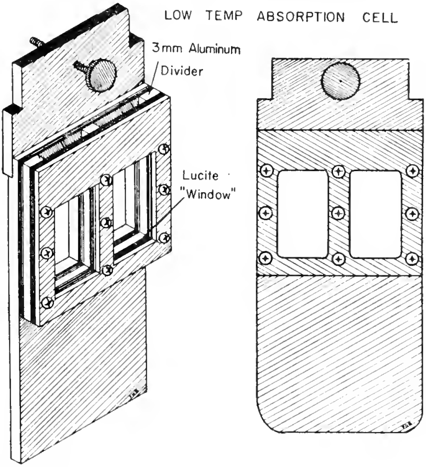

FIG. 1. The absorption cell used for low temperature studies. During the optical measurements, the foot remains in the liquid nitrogen, thus insuring that the temperature of the absorption cell and its contents remains close to −190°C.

The use of low temperature spectrophotometry is essential for the investigation of plant cytochromes for two reasons: (*a*) the relatively low concentration of cytochrome components in plant tissues and in cell-free preparations derived from them, a situation where absorption intensification at low temperature is almost mandatory; and (*b*) the multiplicity of the cytochrome *b* components of close absorption maxima requires the sharpening and definition that is obtained at low temperatures.

In this investigation, four types of difference spectra were used to delineate the cytochromes in particulate preparations. In each case, the spectra were representative of the difference in absorption between an aerated preparation and a preparation treated in one of the following ways:

1. Substrate reduction to different steady states.
2. Reduction with reduced diphosphopyridine nucleotide (DPNH) in the presence of cyanide.

3. Reduction with DPNH in the presence of n-heptyl-8-hydroxy-quinoline-N-oxide (HOQNO).
4. Reduction with sodium dithionite.

It will be seen below that these four methods of treatment coupled to the low temperature absorption spectrophotometry represents a powerful approach to the study of plant cytochromes.

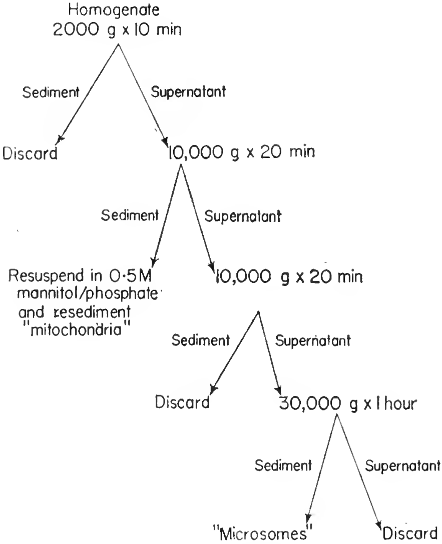
Homogenate
2000 g x 10 min
Sediment
Supernatant
Discard
10,000 g x 20 min
Sediment
Supernatant
Resuspend in 0·5M mannitol/phosphate and resediment "mitochondria"
10,000 g x 20 min
Sediment
Supernatant
Discard
30,000 g x l hour
Sediment
Supernatant
"Microsomes"
Discard

FIG. 2. Preparation and fractionation of plant tissue homogenates. 200 g of plant tissue was ground in 200 ml of 0·5 M mannitol, 0·01 M phosphate (pH 7·3) and 0·1% of cysteine or glutathione and 10^{-3} M versene. The material was either hand-ground in mortar and pestle with sand, or disintegrated in a Waring blender run at a low speed. The ground material was squeezed through muslin, giving the homogenate.

In the following, 'component 558' is used to indicate the cytochrome having an α-absorption band at −190°C in phosphate buffer at 558 mμ, and other cytochromes are indicated by a corresponding notation.

As shown by Okunuki (1939), pollen is a material relatively rich in cytochromes. Dithionite-reduced pollens derived from a variety of sources show three well defined absorption bands when studied with a microspectroscope. The low temperature difference spectrum, resulting from the difference in absorption between a well aerated corn pollen suspension and a dithionite-reduced suspension is shown in Fig. 3. It can be seen that this spectrum shows,

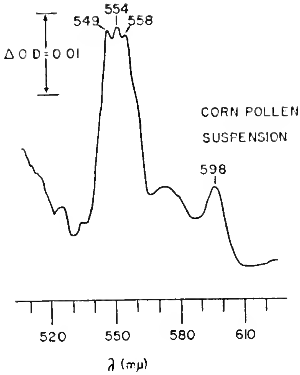

Fig. 3. Difference spectrum (at −190°C) representing the difference in absorption between oxidized and dithionite-reduced corn pollen suspensions.

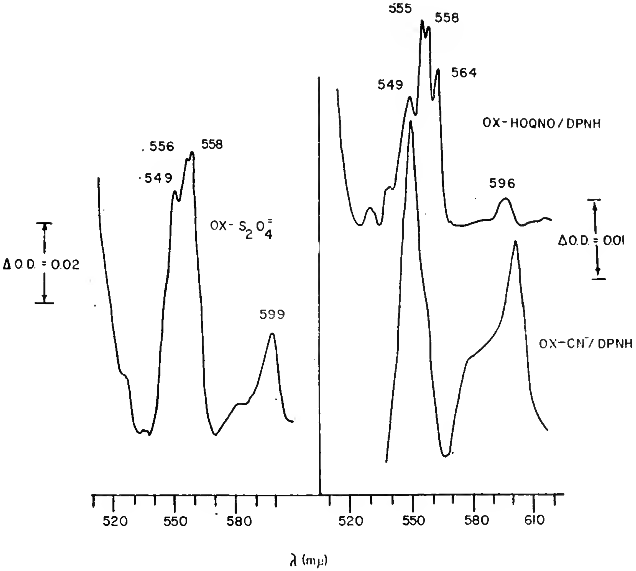

Fig. 4. Difference spectra (at −190°C) obtained from a corn pollen homogenate. The spectra represent the difference in absorption between oxidized homogenates and homogenates reduced with dithionite (left), reduced with DPNH in the presence of HOQNO (upper right) and DPNH reduced in the presence of cyanide (lower right). Note the difference in scale between the spectrum on the left and those on the right. Fresh pollen was supplied by Mr. D. B. Walden of Cornell University, Ithaca, N.Y.

in addition to the $(a + a_3)$ band at 598 mμ and the c band at 549 mμ, two well defined peaks at 558 and 554 mμ. These latter two components are seen as one absorption band when observed with the microspectroscope at room temperature.

In order to delineate the cytochromes of corn pollen more clearly, a homogenate, prepared in 0·1 M sodium phosphate buffer was studied. The low temperature spectra resulting from this study are shown in Fig. 4. Inspection of this figure shows that the dithionite reduced homogenate gives essentially the same spectrum as does the whole pollen suspension. A remarkable effect is observed on reduction of the homogenate by DPNH in the presence of cyanide, under which conditions the 'b' components disappear almost entirely and only cytochromes c and $(a + a_3)$ remain reduced. Such behaviour is very different from yeast and muscle preparations, but has been demonstrated in two aroids *Arum maculatum* (Bendall and Hill, 1956) and *Symplocarpus foetidus* (Chance and Hackett, 1959; Yocum and Bonner, unpublished work).

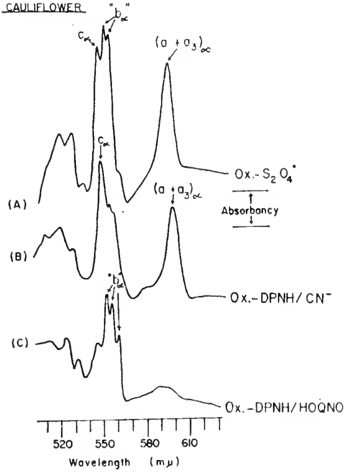

FIG. 5. Difference spectra (at −190°C) obtained from cauliflower mitochondria. Curve *A*, oxidized minus dithionite; Curve *B*, oxidized minus DPNH and cyanide; Curve *C*, oxidized minus DPNH and HOQNO.

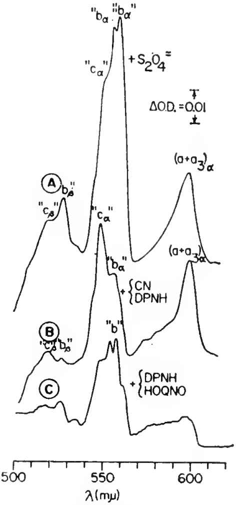

FIG. 6. Difference spectra (at −190°C) obtained from etiolated black valentine bean mitochondria. The spectra represent the difference in absorption between an oxidized mitochondrial suspension and suspensions reduced with A, dithionite; B, cyanide and DPNH; C, DPNH and HOQNO.

Reduction of the corn pollen homogenate in the presence of HOQNO also produces a remarkable spectrum. Under such conditions in the Keilin and Hartree muscle preparations or in yeast suspensions, only cytochrome *b* (at 560 mμ) remains reduced while the $(a + a_3)$ and c components become more oxidized. In the corn pollen homogenate three well defined peaks appear in the presence of HOQNO, these peaks have their maxima at 555, 558 and 564 mμ. Other illustrations of this behaviour toward HOQNO or antimycin A, will be given below. All plant tissues that have been examined so far characteristically show these same three absorption peaks following treatment with HOQNO or antimycin A.

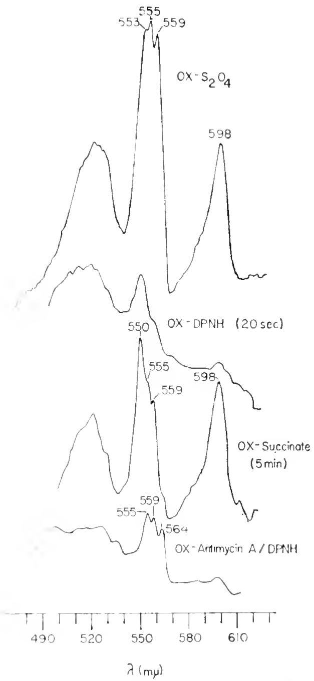

FIG. 7. Difference spectra (at −190°C) obtained from etiolated mung bean hypocotyls. The spectra represent the difference in absorption between oxidized mitochondrial suspensions and suspensions treated as indicated in each curve.

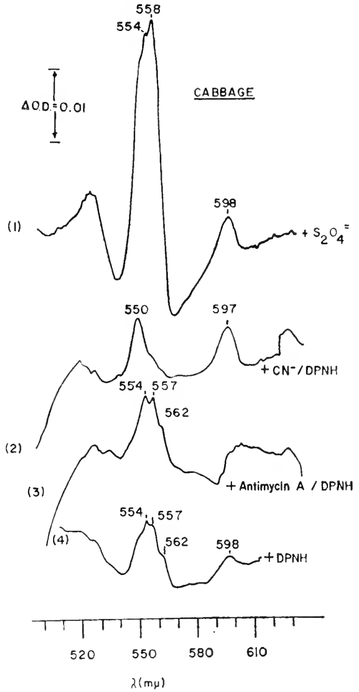

FIG. 8. Difference spectra (at −190°C) obtained from cabbage mitochondria. The spectra represent the difference in absorption between oxidized mitochondrial suspensions and suspensions treated as indicated on each curve.

Figures 5 to 9 show low temperature difference spectra of 'mitochondrial' suspensions derived from various sources. These sources include cauliflower buds, etiolated black valentine bean and mung bean hypocotyls, cabbage and skunk cabbage flowers. All of these preparations show the same characteristic behaviour with HOQNO or Antimycin A and to reduction by DPNH in the presence of cyanide.

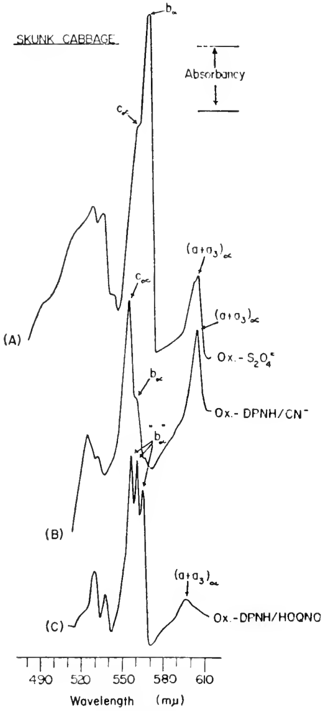

FIG. 9. Difference spectra (at −190°C) obtained from skunk cabbage spadix mitochondria. The spectra represent the difference in absorption between aerobic mitochondrial suspensions and suspensions treated as indicated on each curve.

Reference to the figures showing the low temperature spectra of the various plant preparations shows that there is much more dithionite-reducible cytochrome '*b*' than can be found following substrate reduction, even with anaerobiosis. This behaviour of the '*b*' components is also characteristic of all plant tissues that have been investigated; so far any reasonable explanation of this phenomenon has evaded us. An indication of the magnitude of this difference is shown by the following figures: substrate reduction of mung bean particles; $a + a_3$, 93%; component 558, 43·6% and component 555, 40·8%.

Microsomes

The microsomal fraction, when reduced with dithionite, characteristically shows an asymmetric band of maximum 559 mμ (Fig. 10) or in some instances a two-peak spectrum of maxima 559 and 553 mμ. Reduction of this fraction with DPNH gives a two banded spectrum at 558 and 552 mμ (Fig. 11). The amount of 552 component varies among different plant species.

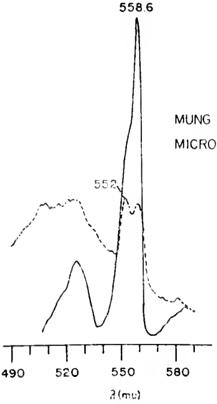

Fig. 10. Difference spectra (at −190°C) obtained from the mung bean hypocotyl microsomal fraction. The curves were obtained from the difference in absorption between aerated microsomal suspensions and suspensions reduced with dithionite (solid line) and DPNH (dashed line).

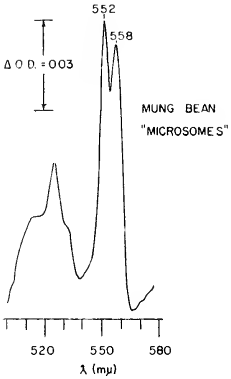

Fig. 11. The difference spectrum (at −190°C) obtained by measuring the difference in absorption between oxidized mung bean microsomes and a suspension of microsomes reduced with DPNH.

Chloroplasts

Cytochromes *f* and b_6, the two haemoproteins that have been described as chloroplast components, are always found in the reduced state; these two components are not easily oxidized chemically (Davenport and Hill, 1954). For this reason, difference spectra were difficult to carry out on isolated chloroplasts. Before entering into a study of the low temperature spectra of isolated chloroplasts it was thought advisable to obtain the low temperature spectrum of cytochrome *f*, the one chloroplast component that has been purified. The extraction and purification of cytochrome *f*, from parsley leaves, was carried out according to Davenport and Hill (1952).

The low temperature absolute absorption spectrum of partially purified cytochrome *f* is shown in Fig. 12. With a sharp maximum at 552 mμ cytochrome *f* can be easily distinguished, at $-190°$, from the other cytochrome components that have been described here. No attempt has been made yet to investigate the absorption properties of the chloroplast *b* component, nor of whole chloroplast suspensions.

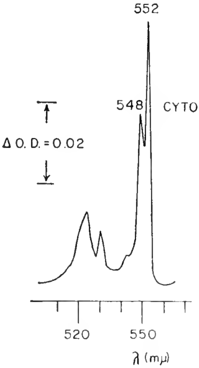

Fig. 12. The absolute absorption spectrum (at $-190°$C) of a cytochrome *f* preparation.

Roots

Particulate preparations were obtained from roots with difficulty and in small yields. Particulate preparations from barley roots showed maxima at 596, 558 and 551 mμ. The spectrum for a particulate preparation derived from bean roots is shown in Fig. 13. It may be seen that reduction with DPNH shows bands corresponding to cytochrome components ($a + a_3$), *b* and *c*; after reduction with dithionite, a very large amount of dithionite-reducible cytochrome appears.

Cyanide Insensitive Respiration

Only brief mention will be made here concerning cyanide-insensitive respiration; for a detailed discussion see Bendall and Hill (1956), Chance and Hackett (1959) and Bonner and Smith (1961). Those tissues which exhibit, characteristically, cyanide-insensitive respiration have been examined in detail for their cytochrome components. 'Mitochondrial fractions', prepared from cyanide-insensitive tissues have been found to contain exactly the same cytochrome components as found in cyanide-sensitive plant tissues. A series of spectra of a particulate preparation from skunk cabbage is shown in Fig. 9. Such particulate preparations are hardly affected in their rate of oxidation of succinate by sodium azide in concentrations as high as 0·01 M. It may be seen that the cytochrome spectrum does not differ in any way from that of cauliflower mitochondria (Fig. 5) which are inhibited fully by relatively small concentrations of sodium azide.

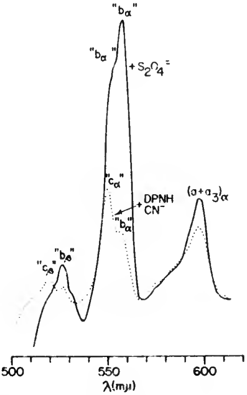

FIG. 13. Difference spectra (at −190°C) obtained from a black valentine bean root particulate preparation. The spectra represent the difference in absorption between aerated particulated suspensions and suspensions reduced with dithionite (solid line) and DPNH (dashed line).

DISCUSSION

In the preceding section a large number of absorption spectra have been presented and now one may well ask what positive contributions to our understanding of plant cytochromes have been made here. How far can one go in delineating the cytochromes of plant tissues? Which specific components are involved in electron transport to oxygen and in the photosynthetic apparatus? What are the immediate problems that now face us? These are important questions and most of the answers are not immediately apparent.

We now have available considerable lore relating to the cytochrome components of plant tissues and this paper adds to this. However, the results presented in this study of plant cytochromes show quite clearly that, in spite of the complexity of the problem, there is an undercurrent of unity. It has been shown here that plant tissues appear to contain some of the same cytochrome components and these components are unique to plants. In short, higher plant tissues possess a common unity of cytochromes components.

It becomes increasingly clear that there is no unity of cytochrome components throughout the biological world; such a unity exists only within each major biological group (cf. Estabrook, this volume, p. 454).

At the present time there is insufficient evidence to assign a localization or a specific name to many of the plant cytochromes. However, one can consider what we know about cytochromes involved in electron transport to oxygen in the cyanide-sensitive portion of the respiratory chain of plant mitochondria.

It would appear that the reaction with oxygen is mediated by cytochrome oxidase and, on the basis of spectral evidence and inhibitor studies, cytochromes *a* and a_3 in plants are very nearly identical to those of animal tissues. This conclusion is by no means a new or surprising one.

The substrate of cytochrome oxidase, cytochrome *c*, is likewise present in plant tissues and in good concentration. Again, the demonstration of cytochrome *c* in plant tissues is by no means new. It is interesting that the plant cytochrome *c*, as delineated most clearly by its reduction upon addition of substrate to mitochondria in the presence of cyanide or azide, has an α-band absorption maximum (−190°C) which is consistently 2 mμ toward the red as compared to animal preparations and pure animal or wheat germ cytochrome *c* solutions, all measured under the same conditions. Such a modest optical variation is not at all surprising. The important thing is that the plant *c* responds very similarly to its animal counterpart in steady state studies. A component corresponding to cytochrome c_1, as defined for animal tissues, has not been found in any plant tissues so far examined.

On the basis of the above discussion we formulate the electron transport system in cyanide-sensitive plant mitochondria as follows:

$$\text{Substrate} \rightarrow \begin{array}{c}\text{DPNH} \rightarrow ?\\ \\ \text{Succinate} \rightarrow ?\end{array} \rightarrow c \rightarrow a \rightarrow a_3 \rightarrow \mathrm{O}_2$$

It is the question marks that are difficult to discuss at our present state of knowledge. The question marks, of course, refer to the baffling nature of the cytochrome *b* components. This problem is further complicated by the fact that possible mitochondrial contamination by plastid fragments, and consequently plastid cytochromes, has not been eliminated.

The spectral data that have been presented in this study show no cytochrome component that can be clearly designated as 'cytochrome *b*'. Keilin and Hartree heart-muscle preparations and yeast suspensions show on substrate reduction in the presence of Antimycin A or HOQNO, one sharp absorption band with a maximum (at −190°C) of 560 mμ. The plant preparations do not reveal a 560 mμ component when similarly treated. Chance and Hackett (1959) also found no evidence for cytochrome *b* in their study of skunk cabbage mitochondria (on reduction by substrate).

In spite of the apparent lack of 'cytochrome *b*' in plant tissues we are still presented with the problem of finding a home for the cytochromes *b* that have been specifically named in plant tissues, viz., cytochromes b_3, b_6 and b_7. The name 'cytochrome b_3' has been given to the microsomal pigment (Martin and Morton, 1953, 1957) while cytochrome b_6 has been localized in plastids (Hill, 1954). Of the named *b* cytochromes in plants we are left with cytochrome b_7 and yet one has to account for the three banded spectrum that characteristically appear on reduction of plant mitochondria in the presence of HOQNO and Antimycin A. It is felt that any attempt to name specifically any of the components *b* that have been presented in this paper would be premature.

There are two characteristics of the *b*-type cytochromes of plant tissues that differ markedly from the cytochrome *b* component of heart-muscle preparations. Steady state substrate reduction studies of substrate reduction in the presence of cyanide both show that plant *b* cytochromes possess a high degree of auto-oxidizability.

The plant tissues and preparations also show an unusually high amount of dithionite-reducible *b*. An important question concerning this dithionite-reducible material is whether it is the same as one of the substrate-reducible components already described, or whether it represents a separate, non-enzymic component. Chance and Hackett (1959) found that the dithionite-reducible component differed in its light absorption characteristics from the substrate-reducible component. Not only were the γ- and α-bands at different positions but the ratio of their intensities also was different, a situation analogous to mammalian tissue preparations (Chance, 1958). The answer to the nature of the dithionite-reducible material is not available at the present time nor is it known if this material represents one or more than one component.

Mention has been made concerning the cytochrome components known to function in electron transport to oxygen in cyanide-sensitive plant mitochondria. While this is not the place for a detailed discussion of cyanide-insensitive respiration, this topic should be considered briefly. Bendall and Hill (1956) have suggested that cytochrome b_7 could act in electron transport to oxygen in the cyanide-insensitive particles extracted from the spadix of *Arum maculatum*. Chance and Hackett (1959), from their study of skunk cabbage, concluded that the time was still premature to consider alternate pathways of electron transport. On the other hand, Bonner and Yocum (unpublished) feel that their evidence with the skunk cabbage does support the alternate pathway hypothesis; although they do agree with Chance and Hackett (1959) that really definite support for an alternate pathway is still lacking.

The evidence presented in this paper has shown the cytochrome components of cyanide-insensitive and cyanide-sensitive tissues to be exactly comparable.

In short, there are no cytochrome components unique to cyanide-insensitive tissues, a fact that requires a further reinterpretation of the alternate pathway hypothesis. Such a reinterpretation has been made by Bonner and Smith (1961) who propose that all tissues possess the capacity for exhibiting cyanide-insensitive respiration. Such a hypothesis presumes two pathways of electron transport to oxygen, perhaps as illustrated in Fig. 14.

Some possible consequences of such a proposal are discussed by Bonner and Smith (1961). However, considerable effort is needed to provide proof that plant tissues can, or cannot, transport electrons to oxygen through a cytochrome system working along with, or alternately to, the *c*, *a*, a_3 system.

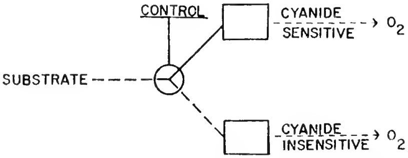

FIG. 14. Hypothetical pathways of electron transport in plant tissues.

Although there has been considerable progress in the last few years in information concerning the cytochromes of plants, there remain enormous gaps in our knowledge.

SUMMARY

1. A survey of the cytochromes in various plant tissues has been carried out utilizing the remarkable sensitivity and resolution of the low temperature divided-beam spectrophotometer.

2. Previous observations, from other investigators showing that plants contain cytochromes $a + a_3$ and *c* have been confirmed and extended.

3. Plant tissues do not appear to contain cytochrome c_1, as defined in animal tissues, nor cytochrome *b*.

4. The cytochromes '*b*' of plants are unusually auto-oxidizable as shown by substrate steady-state reduction of the pigments of mitochondria in the presence and absence of cyanide.

5. Plant mitochondria, when reduced with DPNH in the presence of HOQNO or antimycin-A, show three well-defined absorption maxima at 555, 558 and 564 mμ.

6. The microsomal fraction, derived from various plant tissues, show, on DPNH reduction, a two-banded spectrum of maxima 552 and 558 mμ. Reduction of this same fraction with dithionite results in an asymmetric band of maximum 559 mμ.

7. The cytochromes of roots are exactly comparable to those of other plant parts.

8. The low temperature absorption spectrum of purified cytochrome *f* has been obtained.

9. It is concluded that it is premature to designate names to the cytochrome *b* components described here.

Acknowledgements

The author expresses warm appreciation to Dr. Britton Chance for his enthusiasm and advice during the course of this study. He also thanks Dr. R. W. Estabrook for reading the manuscript, and Mr. N. Meadow and Miss S. Yao for help in the preparation of cytochrome *f*.

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DISCUSSION

Cytochromes c_1 and b_3 of Particulate Components of Plants

By R. K. MORTON (Adelaide)

MORTON: In 1953–1955 in collaboration with E. M. Martin, I investigated some of the haemoproteins of plants. From silver-beet petioles and from wheat roots, mitochondria and microsomal fractions were prepared, the origin and nature of which were investigated by electron microscopy (Hodge, Martin and Morton, *J. Biochem. Cytol.* **3**, 61, 1957). We showed that the microsomal fraction largely originated from two types of lipoprotein membranes which we designated as 'endoplasmic reticulum' and 'golgi zones' respectively. Incidentally, this was the first identification of such structures in higher plants.

When plant mitochondria were heated (to destroy other haemoprotein components), and reduced with ascorbate, cytochrome *c* (α-band, approximately 550 mμ at room temperature) was detected. However, when reduced with DPNH, and to some extent, with succinate, and especially with dithionite, a band appeared close to 554 mμ (at room temperature), which could be readily resolved by low-dispersion microspectroscopy from the cytochrome *c* band at 550 mμ and from the cytochrome *b* band at 560–564 mμ. The band at 554 mμ at first suggested to us contamination with cytochrome *f* (α-band, 555 mμ at room temperature). However, when it was detected even more strongly in mitochondria from plant roots, it was recognized as a component not previously described in plants and called cytochrome c_1 of plant mitochondria (Martin and Morton, *Biochem. J.* **65**, 404, 1957; see also Morton, *Rev. pure appl. Chem.* **8**, 161, 1958). This nomenclature was, at that time (1958), consistent with the report of cytochrome c_1 as a haemoprotein of α-band at approximately 554 mμ (at room temperature) in liver and muscle mitochondria and in other animal tissues. At that time, no function had been allocated to cytochrome c_1; and, indeed, we would be on very dangerous ground today if we attempted to classify cytochromes on a functional basis. Moreover, it appeared undesirable to introduce a new alphabetical designation for every component found in each new tissue examined. Lundegårdh (*Biochim. biophys. Acta* **27**, 355, 1958) confirmed the presence of this component from spectrophotometric studies of plant roots, and showed that its oxidation-reduction state changed during aerobiosis and anaerobiosis. Moreover, in our studies of the effect of inhibitors such as antimycin A (Martin and Morton, *Biochem. J.* **64**, 221, 1956; *Biochem. J.* **65**, 404, 1957) and later of HOQNO and amytal (Wiskich, Morton and Robertson, *Aust. J. Sci.* **13**, 109, 1960) on plant respiration, we observed that cytochrome c_1 was partly reduced in the presence of these respiratory inhibitors.

We therefore tentatively included this cytochrome as a component of the respiratory chain of plant mitochondria (see also Wiskich, Morton and Robertson, *loc. cit.*). This, of course, needs verification. Chance's trapped steady-state procedure (this volume, p. 457) will undoubtedly be valuable for clarifying this question.

In the microsomal fraction, at room temperature only a single oxidizable-reducible haemoprotein was detected, with an α-band, when reduced, at 559 mμ (at room temperature). This new component we first called 'cytochrome b_3' (Martin and Morton, *Nature, Lond.* **176**, 113, 1955) (not wishing to add another cytochrome name to the literature) and later 'cytochrome b_3 (M & M)' (Martin and Morton, 1957, *loc. cit.*) to distinguish this pigment from the component described by Hill and Scarisbrick (*New Phytol.* **50**, 98, 1951).

Cytochrome b_3 of wheat-root microsomes was reduced very rapidly by DPNH and more slowly by TPNH; no other substrate was found. The ferrocytochrome is very rapidly oxidized in air—so rapidly that, by analogy with rates experienced with cytochrome b_5 in microsomes, it appeared possible that a specific cytochrome b_3 oxidase was present (Martin and Morton, 1955, *loc. cit.*). In this connexion, we isolated from silver-beet petiole a very small amount of an oxygen-combining haemoprotein which appeared to have some analogy with the haemoglobins of yeast and fungi described by Keilin and Tissières (see Martin and Morton, 1957, *loc. cit.*) The oxidation of ferrocytochrome b_3 is partly cyanide resistant, suggesting a possible involvement of this cytochrome in the cyanide-resistant respiration of certain plant cells (Martin and Morton, 1955, *loc. cit.*). Lundegårdh (1958, *loc. cit.*) has also detected cytochrome b_3 in plant cells, and Crane (*Plant Physiol.* **32,** 619, 1957) has observed cytochrome b_3 in plant microsomes. We considered that cytochrome b_3 of plant microsomes is closely analogous to cytochrome b_5 of animal microsomes (cf. Martin and Morton, 1955, *loc. cit.*; Bailie and Morton, *Nature, Lond.* **176,** 111, 1955; Morton, 1958, *loc. cit.*) The observations reported here by Bonner have all been made at −190°C and it is therefore difficult to relate the various absorption bands detected by him to the components observed by other workers, and especially to the components which Martin and I designated as cytochrome c_1 and cytochrome b (in mitochondria) and cytochrome b_3 (in microsomes). This difficulty arises because of the variable shifts and variable splitting of the α-absorption bands of the different ferrocytochromes when cooled to −190°C (see Estabrook, this volume, p. 436) and because Bonner's results give difference spectra (reduced minus oxidized component) whereas our results give absorption bands of the reduced component. The broadening and intensification of the absorption at about 560 mμ (at room temperature) on treatment of plant mitochondria with dithionite after addition of substrate under anaerobic conditions is readily observed. We attributed this to modification, probably involving denaturation, of the labile cytochrome b of plant mitochondria by dithionite. In our experience, dithionite readily produces denaturation of labile haemoproteins such as yeast cytochrome c and yeast cytochrome b_2 even when the system is adequately buffered against changes of pH.

Finally, in agreement with Slater, we have suspected that CN^- may combine with cytochrome b of plant mitochondria, thus accounting for the loss of absorption near 560 mμ in the presence of this ligand and substrates.

BONNER: As regards the microsomal component I think that this is a single pigment which, like cytochrome b_5 shows (at −190°C) α_1 and α_2 bands. It differs from cytochrome b_5, as shown in Fig. 10 in my paper, in that, on reduction with DPNH with cyanide present, it no longer retains the double peak but shows a single α-band.

The component with an α-band at 552 mμ (at −190°C) in plants is not reduced by ascorbate. Since cytochrome c_1 of animal tissues is reduced by ascorbate, it does not appear to be identical with cytochrome c_1

ESTABROOK: A further point to be considered in assigning the term cytochrome c_1 to the pigment of plant mitochondria, in addition to its inability to be reduced by ascorbic acid, is the fact that an absorption band characteristic of this pigment appears in the presence of antimycin A. Thus applying the functional definition descriptive of cytochrome c_1 of mammalian systems (cf. Estabrook and Sacktor, *Arch. Biochem. Biophys.* **76,** 509, 1958) one must conclude that this absorption band is *not* associated with a pigment analogous to cytochrome c_1 of mammalian systems.

The Cytochromes of Roots

CHANCE: In the writing of a review on cytochromes in roots with Lucille Smith (Smith and Chance, *Ann. Rev. Plant Physiol.* **9,** 49, 1958), some aspects of Lundegårdh's experimental data on cytochromes b kinetics appeared to be inconsistent with those of other components of the respiratory chain. For example, alternating oxygen and nitrogen in the wheat root bundle causes the absorption bands of cytochromes a, c and b to appear in less than a minute after the aerated medium is replaced by oxygen-free medium. Thereafter large and slow absorbency changes occur in the region of cytochrome b. Similarly in his important experiments on activation of anion respiration, there is a rapid change of cytochrome a_3 and a slower change of cytochrome b which is maximal in about 10 min. The latter change is in fairly good agreement with the time course of the chloride absorption.

In the course of the work on plant cytochromes, it has occurred to Bonner and me that the large amounts of accessory b pigments (see Chance, this volume, p. 476) found in other parts of the plant might also occur in the roots and thereby afford an explanation for the discrepancies of cytochromes a_3, a, c and the large cytochrome b band of Lundegårdh.

Figure 13 of Bonner's paper (p. 493) shows that treatment of bean roots with CN^- and DPNH clearly shows (in a low-temperature difference spectrum) the bands of the cytochrome a_3, a, c, b system in the usual proportions (corresponding roughly to one each of the components). The oxidized minus dithionite spectrum, however, clearly shows a great excess of accessory '*b*' pigments, that completely obscure the *b* and *c* bands of the respiratory components.

This result for bean root suggests that the large amounts of accessory '*b*' pigments may be a general phenomenon and provide an explanation for Lundegårdh's data. In this case we reach the interesting conclusion that the accessory pigment (of *b*-type) is best correlated with the anion transport, thereby providing a haemoprotein of the type required by Davies' theory on this topic.

THE CHEMICAL AND ENZYMIC PROPERTIES OF CYTOCHROME b_2 OF BAKERS' YEAST

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DIXON and colleagues (Bach, Dixon and Keilin, 1942; Bach, Dixon and Zerfas, 1946) first described cytochrome b_2 as a haemoprotein associated with highly purified preparations of lactate dehydrogenase of bakers' yeast. Bach *et al.* (1946) concluded that 'cytochrome b_2 forms an essential part of the enzyme system either as the dehydrogenase itself or as an essential intermediate carrier between lactate and methylene blue.' Subsequently, Morton and co-workers (Appleby and Morton, 1954, 1959a, b, 1960; Armstrong and Morton, 1959) continued studies of cytochrome b_2 to determine the relationship between the haemoprotein and lactate dehydrogenase activity of bakers' yeast. The cytochrome b_2 was isolated as a crystalline deoxyribonucleoprotein which contained equimolecular proportions of protohaem and riboflavin phosphate (Appleby and Morton, 1954, 1959a, b, 1960). Appleby and Morton (1954, 1959b) showed that the flavin group was essential for enzymic activity. Thus cytochrome b_2 was identified as a flavohaemoprotein, a unique type of haematin enzyme with dehydrogenase activity. Boeri and colleagues (Boeri, Cutolo, Luzzati and Tosi, 1955; Boeri and Tosi, 1956), and Nygaard (1958) have confirmed the stoichiometric occurrence of flavin and haem groups in this enzyme.

The isolation procedure (Appleby and Morton, 1954, 1959a; Morton, 1955a, b) involves few treatments and depends on the use of organic solvents (n-butanol and acetone) rather than on ammonium sulphate fractionation and adsorption and elution procedures (Bach *et al.*, 1946; Boeri *et al.*, 1955; Yamanaka, Horio and Okunuki, 1958). Lipid is extracted first from finely-ground, air-dried bakers' yeast by treatment with n-butanol (Morton, 1950, 1955a, b). The cytochrome b_2 and cytochrome c are then extracted from the yeast with lactate solution; the solution is fractionated with acetone at about -5°C, and the cytochrome b_2 is crystallized free of cytochrome c by dialysis to low ionic strength against dilute lactate at about pH 6·8 (Appleby and Morton, 1959a). A yield of 10–20 mg of crystals/kg of dried yeast is consistently obtained and, with only minor modifications, the procedure as described in detail by Appleby and Morton (1959a) has been successfully used for isolation of crystalline cytochrome b_2 from a number of different strains of commercial bakers' yeast obtained in Australia and from other countries. Boeri (personal communication) has recently reported isolation of crystalline cytochrome b_2 from Italian yeast by this procedure. Because of the ready dissociation of flavin from this cytochrome (see later), it is essential to observe the precautions indicated by Appleby and Morton (1959a) in order to avoid modification of the cytochrome during isolation.

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† C.S.I.R.O.

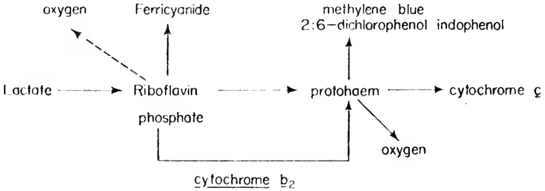

FIG. 1. Scheme for interaction of substrate and acceptors with cytochrome b_2 (Morton, 1955a; Appleby and Morton, 1954, 1955).

Crystalline cytochrome b_2 catalyses the reduction by lactate of ferricyanide, cytochrome *c*, methylene blue, 2:6-dichlorophenol indophenol and oxygen (Appleby and Morton, 1954, 1959b; Armstong and Morton, 1959). Based on detailed chemical and preliminary kinetic studies, it was proposed (Morton, 1955; Appleby and Morton, 1954, 1955) that various hydrogen (or electron) acceptors interact with cytochrome b_2 as shown in Fig. 1. This scheme postulates that reduction of cytochrome *c* and of certain dyes involves an intramolecular hydrogen (or electron) transfer between the flavin and haem groups attached to the one protein molecule, whereas in other systems such as the heart-muscle succinate-cytochrome *c* reductase, transfer takes place between the flavin and haem groups of different proteins, succinate dehydrogenase (flavoprotein) and cytochrome *b* (haemoprotein) respectively (Morton, 1955). The chemical and kinetic evidence presented here further establishes that cytochrome b_2 is a flavohaemoprotein and shows that reduction of cytochrome *c*, ferricyanide and dyes involves different mechanisms.

CHEMICAL PROPERTIES OF CRYSTALLINE CYTOCHROME b_2 AND OF DERIVED PROTEINS

Prosthetic Groups

Maximum lactate dehydrogenase activity (approx. 7750 μmoles of ferricyanide or 5870 μmoles of cytochrome *c* reduced/hr/mg of enzyme at 20°C)

TABLE 1. CHEMICAL ANALYSES OF CRYSTALLINE CYTOCHROME b_2

The material was prepared according to Appleby and Morton (1954, 1959a)

Prosthetic groups and elementary composition (Appleby and Morton, 1959b, 1960; Armstrong and Morton, 1959)			Nucleotide composition (Appleby and Morton, 1960)			Amino acid composition (Appleby, Morton and Simmonds, 1960)		
	g/100 g of dry wt.	moles/mole of haem		g/100 g of dry wt.	moles/mole of haem		g/100 g of dry wt.	moles/mole of haem
Protohaem	0·77	(1·0)	Deoxyribose	2·0	12	Alanine	4·55	39·5
Riboflavin phosphate	0·55	1·0	Ribose	—	0	Amide	9·33	81·0
Total iron	0·077	1·1	Nucleotide P	0·58	15	Arginine	9·70	21·0
Non-haem iron	0·007	0·1	Nucleotide bases:			Aspartic acid	6·85	59·5
Copper	0·016	0·2	Adenine	—	6	½-Cystine	2·12	18·5
Other metals:			Guanine	—	2	Glutamic acid	6·81	59·1
Ca, Al, Zn, Pb, Ag,	Faint trace to doubtful	—	Cytosine	—	2	Glycine	4·77	41·4
			Thymine	—	5	Histidine	2·14	6·2
Mo, Co, Ni	Not detected	—	Uracil	—	0	Isoleucine	6·35	55·2
Total P	0·56	15	Total polynucleotide	6·2	—	Leucine	6·35	55·2
Total N	15·2	—				Lysine	10·42	45·7
Amino acid N	14·2	—				Methionine	<0·3 *	0 *
Total S	0·74	18·5				Phenylalanine	1·61	14·0
						Proline	3·79	32·9
						Serine	3·08	26·5
						Threonine	2·52	22·1
						Tryptophan	0·79	3·4
						Tyrosine	1·60	13·8
						Valine	4·85	42·1

* Less than 0·3% of methionine found by analysis.

is found in twice-recrystallized cytochrome b_2; the activity is constant after further recrystallizations (Appleby and Morton, 1959a, b). Chemical analyses of such material are shown in Table 1.

The absorption spectrum of the pyridine haemochrome of cytochrome b_2 (Bach *et al.*, 1942; Appleby and Morton, 1954, 1959b) and the properties of the isolated haemin (Armstrong and Morton, 1959) show that the haem group is identical with protohaem. The identity of the flavin group with riboflavin phosphate (FMN) was established by paper chromatography and by fluorimetry (Appleby and Morton, 1954, 1959b). The amounts of protohaem (0·77%), riboflavin phosphate (0·55%) and iron (0·077%) all indicate a minimum weight/mole of approx. 80,000 g, with equimolecular proportions of flavin and haem and the absence of non-haem iron.

Mahler and Green (1954) and Mahler (1956) have proposed that the interaction of flavoproteins with cytochrome *c* involves an essential flavin-bound metal (iron, copper, molybdenum or related metals). Green and Beinert (1955) predicted that cytochrome b_2 would contain a non-haem metal group because it reacted with cytochrome *c* and with ferricyanide. However, any mechanism of cytochrome b_2–cytochrome *c* interaction involving such metal groups is not supported by the analyses of crystalline enzyme (Table 1 and Appleby and Morton, 1959b). Morton (1955a) had pointed out that non-haem iron, copper, cobalt, nickel, silver and related metals are either absent or occur only in trace amounts in crystalline cytochrome b_2. Moreover, addition of ferrous sulphate to crystalline cytochrome b_2 causes no stimulation of the activity with cytochrome *c* (Appleby and Morton, 1959b) and dialysis of the non-crystalline cytochrome b_2 of Boeri *et al.* (1955) against chelating agents (*o*-phenanthroline, 8-hydroxyquinoline, or N,N′-dihydroxyethylglycine) causes no loss of enzymic activity (Boeri and Tosi, 1956). This evidence establishes that the interaction of cytochrome b_2 with cytochrome *c* (or other acceptors) cannot involve metals other than the iron of the protohaem prosthetic group and precludes mechanisms of the kind postulated by Green and Beinert (1955) and by Mahler (1956). It should be noted, however, that Boeri and Tosi (1956) found 8 g atoms and Boeri and Cutolo (1958) 1 g atom of non-haem iron/mole of haem in non-crystalline cytochrome b_2 and Boeri and Tosi (1956) have proposed that these iron atoms have an essential function in the enzymic reduction of cytochrome *c*. On the basis of the findings of Boeri and colleagues, Mahler (1956, p. 224) has described yeast lactate dehydrogenase as a metalloflavoprotein. However, the evidence strongly suggests that the non-haem iron found by Boeri and co-workers is an impurity in their preparations of cytochrome b_2.

Crystalline cytochrome b_2 sediments at pH 6·8 and at 0°C as a single component (sedimentation constant, $S^0_{20,W}$, 8·48*s*). The enzyme may exist as a dimer with two moles each of riboflavin phosphate and of protohaem/mole of enzyme. Prolonged electrophoresis at pH 6·6, 7·05 and 8·8 also indicates that crystalline cytochrome b_2 contains no protein other than the flavohaemoprotein (Appleby and Morton, 1960). The amino acid composition (Table 1) is rather similar to that of pig-heart diphosphopyridine nucleotide (DPN)-diaphorase (a flavoprotein) and to that of cytochrome *c* (a haemoprotein) (Morton, 1958; Appleby, Morton and Simmonds, 1960). The only unique feature is the apparent absence ($< 0{\cdot}3\,\%$) of methionine which usually accounts for 0·5 to 5% of the dry weight of enzymes and other proteins. This feature of the amino acid composition thus supports the purification studies and the physicochemical analyses in showing that only one protein component is present in the crystalline enzyme.

The preparation from yeast of a crystalline haemoprotein which has an absorption spectrum resembling (between 400 and 600 mμ) that of cytochrome b_2 has been reported by Okunuki and colleagues (Yamashita *et al.*, 1957). The preparation has no lactate dehydrogenase activity and contains no flavin group (Yamanaka *et al.*, 1958). This could imply the existence in yeast of two distinct proteins, a flavoprotein lactate dehydrogenase, and a haemoprotein which acts as the specific hydrogen (or electron) acceptor for the flavoprotein dehydrogenase. The homogeneity of crystalline cytochrome b_2 of Appleby and Morton (1954, 1959a) having lactate dehydrogenase activity, however, does not support this view. Moreover, it is significant that equimolecular amounts of flavin and of haem are found in preparations of enzymically-active cytochrome b_2 obtained by such different procedures as (*a*) extraction with butanol-saturated lactate, fractionation with acetone and crystallization at low ionic strength (Appleby and Morton, 1954, 1959a); (*b*) fractionation of a yeast autolysate with ammonium sulphate and treatment with calcium phosphate gel (Boeri *et al.*, 1955); and (*c*) grinding of yeast cells, fractionation with acetone and chromatography on a diethylaminoethyl (DEAE)-cellulose column (Nygaard, 1959). Yamashita *et al.* (1957) used autolysis for 7 days at pH 8·5 followed by fractionation with ammonium sulphate and chromatography on a cation-exchange resin (Duolite CS-101) buffered at pH 5·0. Experience with crystalline cytochrome b_2 (Appleby, 1957; Appleby and Morton, 1959b) strongly suggests that the conditions of prolonged autolysis and of resin-column treatment used by Yamashita *et al.* (1957) would cause dissociation of the flavin prosthetic group of cytochrome b_2 and consequent loss of dehydrogenase activity. It is therefore concluded that the haemoprotein obtained by Yamashita *et al.* (1957) is a modified protein derived from cytochrome b_2. This conclusion is supported by kinetic studies as presented here.

The Flavin–Protein Bonds

Loss of enzymic activity of cytochrome b_2 is frequently accompanied by the appearance of flavin fluorescence (Appleby and Morton, 1954; Boeri *et al.*, 1955); this is prevented, and activity is protected by exclusion of oxygen, by the presence of ethylenediaminetetra-acetate (EDTA) and by substrate (lactate) (Morton, 1955a; Appleby and Morton, 1959b). Under some conditions autoxidation leads to formation of H_2O_2, since catalase partially, but not completely, protects against inactivation (Table 2). The formation of H_2O_2 under these conditions may indicate a slow reaction of oxygen with the flavin group (see Fig. 1), or, more likely, autoxidation of some dissociated flavin since free riboflavin phosphate may act as an acceptor with cytochrome b_2. Oxygen also reacts slowly with the haem group (Fig. 1 and *vide infra*).

Protection by EDTA is due to chelation of metals, such as copper, which

TABLE 2. PROTECTION OF ENZYMIC ACTIVITY OF CRYSTALLINE CYTOCHROME b_2 BY CATALASE

Crystalline cytochrome b_2 (40 μg/ml) in M sodium lactate at pH 6·8 was held aerobically with and without added crystalline catalase (about 100 μg/ml). After storage, some of the material was further treated with hydrogen peroxide. Activities were determined at 18°C and at pH 8·0. The results are from Appleby and Morton (1959b).

Treatment	Activities (μmoles of ferricyanide reduced/hr/ml of enzyme)		
	Initial	Without catalase	With catalase
Storage, 4 days, 0°C	248	94	230
Stored material treated with 0·2 mM H_2O_2 for 10 min at 0°C	230	78	198

TABLE 3. INHIBITION OF ENZYMIC ACTIVITY OF CYTOCHROME b_2 BY Cu^{++} IONS AND PROTECTION BY ETHYLENEDIAMINE TETRA-ACETATE (EDTA)

Crystalline cytochrome b_2 in 0·5 M sodium lactate at pH 6·8 was held aerobically at 0° for 18 hr, with and without the additions indicated. Activities are expressed as μmoles of ferricyanide reduced/hr/ml of enzyme at 18°C and at pH 8·0. The results are from Appleby and Morton (1959b).

Treatment		Activity
Concentration of $CuSO_4$ (μM)	Concentration of EDTA (μM)	
0	0	119*
10	0	10
10	10	97
10	100	195

* With anaerobic storage, activity 179.

catalyse autoxidation. The inactivating effects of copper salts, and the protection by EDTA, is shown by Table 3.

Both the metal-catalysed and the direct autoxidations cause appearance of fluorescence due to dissociation of the FMN group of cytochrome b_2 (Appleby and Morton, 1959b). Since copper salts readily catalyse oxidation of many thiols (see Barron, 1951), the probable involvement of thiol groups is indicated.

In the presence of substrate, *p*-chloromercuribenzoate (10^{-2} to 10^{-4} M) strongly inhibits lactate dehydrogenase activity (Appleby and Morton, 1954; Boeri *et al.*, 1955) whereas mono-iodoacetate (10^{-2} M) under similar conditions has little effect (Boeri *et al.*, 1955). Hence some thiol groups of the enzyme are protected from alkylation, although readily reacting with the powerful mercaptide-forming reagent. Inhibition by *p*-chloromercuribenzoate causes appearance of strong fluorescence, indicating displacement of the riboflavin phosphate group by this reagent (Armstrong, Coates and Morton, 1960).

Since the active enzyme itself is not fluorescent, the imino group of the iso-alloxazine ring is probably bonded to a group of the protein, and it seems likely that there is strong hydrogen-bond formation between the imino group and an ionised thiol group of the protein. There are 18 S-containing residues/mole of haem (Table 1). Many of these may occur as cystine, and relatively few as cysteine. Flavin fluorescence also appears under acid ($<$ pH 4) and alkaline ($>$ pH 9) conditions, under which conditions activity with ferricyanide and with cytochrome *c* is greatly decreased (Appleby, 1957; Appleby and Morton, 1959b).

The enzyme is considerably protected by lactate, and, at the same time, there is marked reduction in appearance of flavin fluorescence. The ready appearance of flavin fluorescence in the oxidized enzyme, and protection by lactate and EDTA, suggest that the dissociation constant of the flavin group of oxidized cytochrome b_2 is very much greater than in the reduced cytochrome. The dissociation constant of the oxidized form of the 'old yellow enzyme' is over 100 times greater than that of the reduced form (Nygaard and Theorell, 1955; Vestling, 1955). If the flavin group is bound by thiol groups as proposed, oxidation of thiol groups after dissociation of the flavin would prevent recombination of the flavin and haemoprotein. The dissociation of flavin from cytochrome b_2 is therefore strictly reversible only under conditions which prevent oxidation of the thiol groups of the apoproteins. The substrate probably protects enzymic activity by maintaining the FMN group in the reduced state and thus lowering the dissociation from the apoprotein.

The Haem–Protein Link

As in other cytochromes, the haem group is strongly bound by the apoprotein, but may be split by treatment with acid–acetone (Armstrong and

Morton, 1959). The positions and extinction coefficients of the α, β and γ-bands of reduced cytochrome b_2 (Fig. 2 and Table 4) are very close to those of pyridine protohaemochrome and suggest that positions 5 and 6 of the iron atom are co-ordinated with strongly basic groups. Since Ehrenberg and Theorell (1955) found that the α-NH$_2$ group of glycylglycine and the ε-NH$_2$ group of lysine do not readily co-ordinate with the iron of either ferri- or ferro-protoporphyrin, it seems probable that at least one of the groups involved in co-ordination is an imidazole nitrogen of a histidine residue. There are 6 such residues/mole of haem (Table 1).

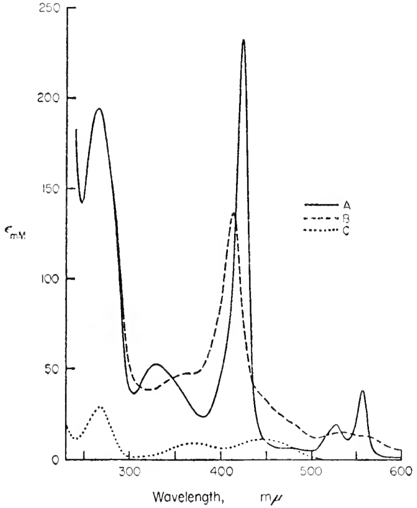

FIG. 2. Absorption spectra of cytochrome b_2. Crystalline cytochrome b_2 (deoxyribonucleoprotein) at pH 6·8:

(*A*) reduced with lactate;
(*B*) oxidized;
(*C*) oxidized riboflavin phosphate at a concentration equivalent to that of the flavin in cytochrome b_2.

The absorption spectra of lactate-reduced cytochrome b_2 at pH 4·7, 6·5 and 10·0 are shown in Fig. 3. At pH 10·0, the solution was strongly fluorescent, indicating extensive dissociation of the riboflavin phosphate, but there was no detectable shift in the positions of the α-, β- or γ-bands of the haemochrome. However, as compared with the spectrum at pH 6·5, the spectrum at pH 4·7 and 10·0 shows a decrease in the ratio of $E_{\gamma\text{-band}}/E_{\delta\text{-band}}$. The increased absorption in the region 440–500 mμ at pH 10·0 as compared with that at pH 6·5 probably reflects the dissociation of the riboflavin phosphate group.

TABLE 4. PRINCIPAL ABSORPTION BANDS OF CRYSTALLINE CYTOCHROME b_2 AND OF ITS DERIVATIVES

Wavelengths (λ) are mμ, extinction values (E) are given for mM concentration of protein-bound haem; some are calculated values, and may be subject to slight revision. Values are given for neutral pH at room temperature unless otherwise stated.

A. Bands in reduced compounds

Compound		α	β	(?)	γ	δ	(?)
Crystalline[1] cytochrome b_2	λ	556·5	528	460	423	330	265
	E	38·8	18·6	8	232	52·2	199
Nucleotide-free[2] cytochrome b_2	λ	557	528	460	423	330	268
	E	36	25		224		135
Haemoprotein[3] derivative	λ	557	528	460	422		
	E	38	22	6	200		
Transient intermediate[4] (lactate complex)	λ	567	533				
Nucleotide-free cytochrome b_2 (at −190°C)[5]	λ	557·5 (α_1) 552·5 (α_2)	532·5 (β_1) 525·0 (β_2)				

B. Bands in oxidized compounds

Compound		α	β	(?)	γ	δ	(?)
Crystalline[1] cytochrome b_2	λ	560*	530*	460†	413	359	275
	E	10	14	25	137	47·6	198
Nucleotide-free[2] cytochrome b_2	λ	560*	530*	460†	412	359	
	E						
Haemoprotein[5] derivative	λ	563*	530*	460†	411	359	278
	E	8		18	140	30	106

* Broad band. † Shoulder only.

[1] Crystalline cytochrome b_2 of Appleby and Morton (1954, 1959a) (flavohaemoprotein containing deoxyribosepolynucleotide).

[2] Nucleotide-free cytochrome b_2 (Boeri *et al.*, 1955; Armstrong and Morton, 1959) (flavohaemoprotein).

[3] Enzymically-inactive haemoprotein derivative of cytochrome b_2 (Yamashita *et al.*, 1957, 1958). The extinction values given for this compound are relative only and may be subject to revision.

[4] Transient intermediate ('lactate'-cytochrome b_2 complex of Appleby and Morton (1959b), see text).

[5] Nucleotide-free cytochrome b_2 (Boeri *et al.*, 1955) at temperature of liquid air (Lindenmayer and Estabrook, 1958).

Table 4 compares the extinction values and positions of the principle absorption bands of reduced crystalline cytochrome b_2, of nucleotide-free cytochrome b_2, and of the flavin-free haemoprotein derived from cytochrome b_2. In the fully reduced enzyme, the flavin appears to have little influence on the contribution of the haem group to the absorption spectrum (see also Fig. 2). The high extinction values of the principal absorption bands of cytochrome b_2 as compared with those of other cytochromes of group *B* (see Morton, 1958, Table 5) therefore does not seem to be due to interaction between the flavin and haem prosthetic groups. Moreover, if there were direct interaction between the resonant systems of the fully reduced protohaem and flavin groups, the positions of the absorption bands of cytochrome

b_2 might be expected to shift as compared with those of pyridine protohaemochrome. Table 4 shows that this is not so.

However, when a freshly-prepared pellet of crystalline cytochrome b_2 is washed and dissolved in 0·5 M NaCl, containing 0·1 mM EDTA at pH 6·8 and at 0°, and then gently shaken in air, the intense absorption bands of the reduced enzyme at 556·5 and 527 mμ slowly fade and are replaced by somewhat weaker bands at 567 and 533 mμ (Appleby, 1957; Appleby and Morton, 1959b). These bands fade completely on further oxidation. Addition of further lactate, or of $Na_2S_2O_4$, causes immediate re-appearance of the bands of the reduced enzyme, at 556·5 and 527 mμ. The oxidized cytochrome b_2 has a typical ferrihaemochrome-type spectrum, with a weak, broad band between 530 and 560 mμ (Fig. 2). Appleby and Morton (1959b) therefore considered that the bands at 567 and 533 mμ were those of the enzyme at an intermediate state of oxidation. If there was formed a stabilized flavohaemoprotein-lactate complex, in which the resonance pathway is lengthened by interaction of the π-electron systems of the flavin and haem prosthetic groups, it would be expected that the absorption bands would be at longer wavelengths as compared with those of the fully-reduced flavohaemoprotein.

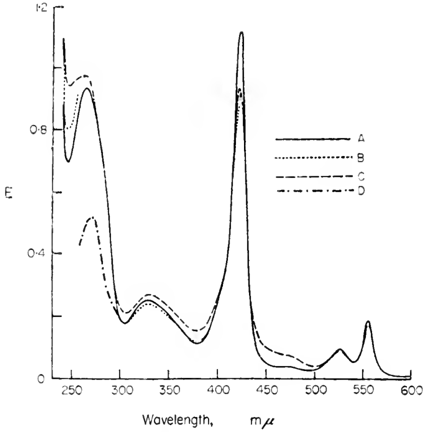

FIG. 3. Lactate-reduced crystalline (deoxyribonucleoprotein) material at pH 6·5 (*A*), 4·7 (*B*) and 10·0 (*C*). Curve *D* is of lactate-reduced nucleotide-free flavohaemoprotein at pH 6·8.

Our interpretation of the transient appearance of absorption bands at 567 and 533 mμ is that they are indeed the bands of an intermediate flavohaemoprotein-lactate complex, and that they are due to the influence of lactate and flavin on the resonance system of the haem group. The bands at 567 and 533 mμ are unlikely to be due to a semi-quinone form of the flavin group. Such semi-quinone intermediates, observed in some flavoproteins, have a rather weak band at about 560 mμ but show no band near 533 mμ (see Beinert, 1957) whereas a well defined two-banded spectrum is observed with the transient form of cytochrome b_2. This also suggests that the haem, as well as the flavin group, is functional in the lactate dehydrogenase activity of cytochrome b_2. Kinetic studies, as discussed below, provide evidence consistent with this interpretation.

The Deoxyribose Polynucleotide Component of Crystalline Cytochrome b$_2$ *and the Nucleotide–Protein Link*

The crystalline cytochrome b_2 of Appleby and Morton (1954, 1959a) has a prominent absorption band at 265 mμ (see Table 4 and Figs. 2 and 3). This is due to a deoxyribose polynucleotide, which is not dialysable, and which is about 6% of the dry weight of the material (Appleby, 1957; Morton, 1955a, 1958; Appleby and Morton, 1960). It contains about 15 nucleotide residues/mole of haem. Re-crystallized cytochrome b_2 contains no ribose or uracil (Table 1). The molecular ratio of $\frac{\text{adenine} + \text{thymine}}{\text{guanine} + \text{cytosine}}$ in the polynucleotide is 2·60 and in yeast deoxyribonucleic acid is 1·79 (Appleby and Morton, 1960; Montague and Morton, 1960). The polynucleotide is a unique and integral component occurring in constant amount in different batches of crystalline cytochrome b_2.*

More recently, Nygaard (1958) has found dialysable and non-dialysable ribonucleotides associated with non-crystalline cytochrome b_2. This type of material remains in the mother liquor when cytochrome b_2 is crystallized (Appleby and Morton, 1960).

The deoxyribose polynucleotide is not essential for lactate dehydrogenase activity of cytochrome b_2. It may be split from the enzyme by digestion with deoxyribonuclease; by precipitation of the protein with ammonium sulphate; and by electrophoresis at a suitable pH (Appleby and Morton, 1960). The function of this component is not known. Uptake of ^{32}P-labelled inorganic phosphate during lactate oxidation is not catalysed by cytochrome b_2 (Appleby and Morton, 1960).

ENZYMIC PROPERTIES OF CRYSTALLINE CYTOCHROME *b*$_2$

Substrate Specificity

Armstrong and Morton (1959) have shown that crystalline cytochrome b_2 catalyses reduction of ferricyanide by a number of α-hydroxy-monocarboxylic acids (Table 5). Of the compounds tested, lactate is clearly the most effective substrate. Table 5 also shows that a number of other hydroxy acids strongly inhibit oxidation of lactate; D-malate is particularly inhibitory. Neither reduced di- or tri-phosphopyridine nucleotide (Yamashita *et al.*, 1957; Yamanaka *et al.*, 1958) nor malate (Yamanaka *et al.*, 1958; Yamashita *et al.*, 1958) are substrates of the crystalline enzyme.

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* *Added in proof.* The polynucleotide has been obtained as homogeneous material of molecular weight approx. 10,000 (Montague and Morton, 1960).

The results show that there is a high degree of specificity at the substrate binding site.

TABLE 5. SUBSTRATE SPECIFICITY OF CRYSTALLINE CYTOCHROME b_2

Relative activities were determined spectrophotometrically by the rate of reduction of potassium ferricyanide at pH 8·0 in 0·033 M sodium pyrophosphate-HCl buffer containing 10^{-5} M EDTA. Relative inhibition was determined under generally similar conditions with DL-sodium lactate (0·1 M) as substrate. Where appropriate the DL-compounds were used in all cases, unless otherwise specified, at 0·1 M concentration. Results of Armstrong and Morton (1959).

Compound	Relative rate (%) of reduction of potassium ferricyanide	Relative inhibition (%) of oxidation of lactate
Glycollate	3	21
Lactate	100	0
Malate	0	95
D-malate[1]	—	86
L-malate[2]	0	53
Glycerate	0	40
L(+)-tartrate[3]	5	0
TPNH	0	0
Mandelate	—	72
Hydroxy-malonate	0	8
α-hydroxy-glutarate[3]	0	7
α-hydroxy-n-butyrate	30	45
α-hydroxy-iso-butyrate	0	32
α-hydroxy-n-caproate	18	65
α-hydroxy-iso-caproate	17	51
α-hydroxy-iso-valerate	0	76

[1] D-malate (commercially prepared material), 0·01 M concentration.

[2] L-malate (commercially prepared material), 0·05 M concentration.

[3] L(+)-tartrate and L(+)-α-hydroxy-glutarate, 0·05 M concentration.

Reactivity of Crystalline Cytochrome b$_2$ *with Oxygen, Ferricyanide, Cytochrome* c *and Dyes*

Crystalline cytochrome b_2 containing very small amounts of lactate is slowly oxidized aerobically. At pH 7·4 and at 0°C in the presence of 0·5 M NaCl, 0·1 mM EDTA, and 0·01 M Tris (2-amino-2-hydroxymethylpropane-1:3-diol)–HCl buffer, the rate of oxidation was equivalent to the oxidation of 80 moles of lactate/hr/mole of haem (Appleby and Morton, 1959b). With prolonged exposure to air, small amounts of H_2O_2 may be formed (*vide supra*).

With 0·5 M lactate and mM EDTA, cytochrome b_2 is only slowly oxidized by air. Under these conditions, the oxygen probably reacts slowly with the haem group rather than with the flavin group, so that H_2O_2 is not formed (see Fig. 1).

However, by contrast, Yamashita *et al.* (1958) have found that the flavin-free haemoprotein derivative of cytochrome b_2 'has a remarkable autoxidizability'. Other typical cytochromes of group *B*, such as cytochrome b_5, also are fairly rapidly autoxidizable (Strittmatter and Velick, 1956; Morton, 1958). If the crystalline haemoprotein derived from cytochrome b_2 by the procedure of Yamashita *et al.* (1957) is not seriously denatured, it would appear that the presence of the flavin group modifies the reactivity of oxygen with the haem group of intact cytochrome b_2. This is further evidence in support of the close juxtaposition of the two prosthetic groups on the one apoprotein in the intact cytochrome b_2.

As compared with the rates with oxygen, the cytochrome b_2-catalysed reduction of ferricyanide, cytochrome *c* and dyes is very much greater. The optimum pH values for reduction of these acceptors vary somewhat according to the purity of the enzyme (Appleby and Morton, 1959b). For crystalline cytochrome b_2, the optimum pH values are: ferricyanide, 8·0; cytochrome *c*, 7·0; methylene blue, 6·8.

The relative rates of reduction of ferricyanide, heart-muscle cytochrome *c* and methylene blue are substantially the same for crystalline cytochrome b_2 as for an extract of dried yeast (Table 6). This shows that the reactivity of

TABLE 6. COMPARATIVE LACTATE DEHYDROGENASE ACTIVITIES OF A CRUDE EXTRACT OF BAKERS' YEAST AND OF CRYSTALLINE CYTOCHROME b_2 WITH DIFFERENT ACCEPTORS

For the crude extract, dried yeast was extracted with 0·15 M NaCl at 38°C for 1 hr and then centrifuged at $10^5 \times g$ for 1 hr at 5°C. The clear supernatant, containing 2·6 mg of protein N/ml, was used. Activities were determined under comparable conditions: these are sub-optimal in respect of cytochrome *c* concentration and pH (see Appleby and Morton, 1959a). Activities are expressed as units/mg (μmoles of acceptor reduced/hr/mg of protein at 18–20°C). Results of Appleby and Morton (1959a).

Hydrogen acceptor	Concentration (mM)	pH	Lactate dehydrogenase activities			
			Yeast extract		Crystalline cytochrome b_2	
			Units/mg	Relative activity	Units/mg	Relative activity
Ferricyanide	0·5	8·0–8·4	36	100	7750	100
Cytochrome *c*	0·02	6·0–7·0	6·6	18	1730	22
Methylene blue	0·08	5·2	2·9	8	1030	13

the enzyme with these acceptors is not changed during the purification procedure, and the enzyme is unlikely to have been modified during isolation.

However, prolonged storage of cytochrome b_2, under aerobic conditions, even in the presence of EDTA, causes partial or complete dissociation of the flavin group and a corresponding decline in activity with ferricyanide (Appleby, 1957; Appleby and Morton, 1959b). After this type of modification, some of the haem of the preparation is only slowly reducible by lactate (Table 7).

TABLE 7. EFFECT OF AEROBIC STORAGE ON THE ENZYMIC ACTIVITY OF CYTOCHROME b_2

Crystalline cytochrome b_2 was suspended in water and held aerobically at 0°C. Portions of the suspension were diluted into 0·5 M sodium lactate containing 10^{-4} M EDTA at pH 6·8 and at 0°C at the times indicated. Activity with ferricyanide was measured immediately. The position and extinction of the Soret band was measured after 10 min at 0°. (From Appleby and Morton, 1959b.)

Storage time	Relative activity with ferricyanide (%)	Position of Soret band ($\lambda_{\max}$ in mμ)	Relative amount of total haem reduced by lactate (%)
0	100	424	100
10 min	55	423·5	87
18 hr	21	419	43

The considerable autoxidizability of the flavin-free derivative of cytochrome b_2, and the slow reduction of inactive cytochrome b_2 by active enzyme (Appleby and Morton, 1954; Yamashita *et al.*, 1958), are complicating features in dealing with mixtures of inactive and active cytochrome b_2. Stepwise modification of crystalline cytochrome b_2, by (a) removal of the polynucleotide, and (b) subsequent dissociation of the flavin, is being carried out and the physicochemical, chemical and enzymic properties of the derivatives of the crystalline enzyme are being studied. However, intact crystalline cytochrome b_2 has been used throughout the kinetic studies described below.

KINETICS OF LACTATE OXIDATION CATALYSED BY CYTOCHROME b_2

Differences between the mechanisms of reduction of ferricyanide and of cytochrome c by cytochrome b_2 appeared likely when it was found (Boeri *et al.*, 1956; Appleby, 1957; Appleby and Morton, 1959b) that reduction of ferricyanide was apparently of zero order, and of cytochrome c of first order. Further kinetic studies by Armstrong and Morton (1959) were carried out with a double-beam recording spectrophotometer (Optica CF 4). Extinctions (E) were measured at 420 mμ (ferricyanide), 550 mμ (cytochrome c) or 600 mμ

(2:6-dichlorophenolindophenol, DCIP), with 0·03 M sodium pyrophosphate-HCl buffer at pH 8·0, 10^{-5} M EDTA, 0·1 M DL-lithium lactate, and crystalline cytochrome b_2 (1 to 2×10^{-8} M). With ferricyanide, plots of E versus time (T) were non-linear, except for short time periods (up to 2 min) at high ferricyanide concentrations (> 1·4 mM). The results conform approximately with those expected for a first-order reaction.

Essentially similar behaviour was observed with heart-muscle cytochrome *c* as acceptor. With these acceptors, the first-order rate constant (k) was estimated and different K_m values for the acceptors were estimated from plots of $1/kS$ against $1/S$.

With DCIP, however, the reaction was substantially of zero order over the range 0·22–0·022 mM dye. Table 8 summarizes the apparent kinetic parameters for the various acceptors.

In Table 8, the values given are called 'apparent kinetic parameters', since the concentration of lactate was not varied, and there is a possibility that the meaning of K_m and V given in Table 8 do not have the usual significance accorded to them. The true kinetic parameters for the reaction with heart-muscle cytochrome *c*, as calculated from the studies reported below (mechanism *III*) are given in Table 9. These values are not so very different from the apparent K_m and apparent V as given in Table 8.

TABLE 8. VALUES OF APPARENT K_m AND APPARENT V* FOR REACTION OF CYTOCHROME b_2 WITH VARIOUS HYDROGEN ACCEPTORS AT pH 8·0 AND 30°C

Results of Armstrong and Morton (1959); for experimental conditions, see text.

Acceptor	Concentration range for acceptor (mM)	V (μmoles/l./min)	V/e (moles/l./min/ *m* mole of haem)	K_m (μM)
Ferricyanide	0·13 –3·42	121	12	190
Cytochrome *c*	0·005–0·17	270	11	44
DCIP	0·022–0·22	67	7	34

* V, maximum initial velocity at infinite concentration of reactants.

TABLE 9. KINETIC PARAMETERS FOR THE REACTION OF CYTOCHROME b_2 WITH CYTOCHROME *c*

Results of Armstrong and Morton (1959).
Determinations were carried out at pH 8·0 and 30°C.
For other experimental conditions, see text.

Range of Acceptor concentration (mM)	Range of Lactate concentration (mM)	V (μmole/l./min)	V/e (moles/l./min/*m* mole of haem)	K_m (μM of L(+)-lactate	K (cytochrome) (μM)
0·052–1·74	0·1–5·0	284	11·3	308	47

Kinetic values vary according to conditions of determination. The conditions used by Appleby and Morton (1954) were suboptimal, as indicated by subsequent kinetic studies (Appleby, 1957; Appleby and Morton, 1959a, b). The procedure for purification and crystallization of cytochrome b_2 has not been altered since 1953, when the first crystalline preparations were obtained. The recent statement by Nygaard (1959), which suggests that the original preparations of Appleby and Morton had low activity as compared with preparations obtained subsequently, is misleading. The values given here and by Appleby and Morton (1959a, b) are higher than those reported initially (Appleby and Morton, 1954) only because of different methods of determination of activity. All values are valid and refer to the activities of crystalline cytochrome b_2 under the conditions specified.

Further information concerning the mechanism of action of cytochrome b_2 with different acceptors has been obtained from kinetic studies in which the concentration of lactate (S) was varied at several different concentrations of hydrogen acceptor (A), in order to differentiate between several possible enzymic mechanisms.

Oxidations catalysed by flavin-linked enzymes may conform to any one of at least three different types of mechanisms. Using a simplified notation (in place of the detailed notation shown in the Appendix (p. 521)), these mechanisms and the corresponding steady state rate equations are as follows.

I. Oxidation of a binary complex by acceptor (cf. Chance, 1953).

$$\mathrm{E + S \rightleftharpoons ES}$$
$$\mathrm{ES + A_{ox} \rightarrow E + P + A_{red}}$$

(where $\mathrm{A_{ox}}$ and $\mathrm{A_{red}}$ represent the oxidized and reduced forms of the acceptor respectively; other symbols follow usual conventions).

Here
$$v = \frac{V}{1 + \dfrac{K_m}{[\mathrm{S}]}}$$

Both V and K_m are functions of the acceptor concentration [A], and have no theoretical finite limit; $\dfrac{K_m}{V}$ is not generally constant (see Appendix).

II. Formation of (*a*) *two or more binary complexes or* (*b*) *a ternary complex* (see Alberty, 1956).

(*a*)

$$\mathrm{E + S \rightleftharpoons ES}$$
$$\mathrm{ES + A \rightleftharpoons EP + H_2A}$$
$$\mathrm{EP \rightleftharpoons E + P}$$

or (*b*)

$$\mathrm{E + S \rightleftharpoons ES}$$
$$\mathrm{ES + A \rightleftharpoons ESA}$$
$$\mathrm{ESA \rightleftharpoons E + P + H_2A}$$

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_{AS}}{[A][S]} + \frac{K_m}{[S]}}, \qquad V_x = \frac{V}{1 + \frac{K_A}{[A]}} \quad \text{and} \quad K_{m_x} = \frac{K_m + \frac{K_{AS}}{[A]}}{1 + \frac{K_A}{[A]}}$$

These mechanisms cannot be distinguished between by steady state kinetics. V_x and K_{m_x} are the values obtained from Lineweaver–Burk plots at any acceptor concentration $[A]_x$, and approach limit values of V and K_m as $[A]_x$ approaches infinity. The slope of the Lineweaver–Burk plots, K_{m_x}/V_x depends on $[A]_x$. It will be noted that V_x is a hyperbolic function of $[A]_x$, while K_{m_x} contains terms which are hyperbolic and inverse functions of $[A]_x$.

III. Formation of an intermediate reduced form of enzyme (see Alberty, 1956).

$$E + S \rightleftharpoons ES$$
$$ES \rightarrow E + P$$
$$E + A_{ox} \rightarrow E + A_{red}$$

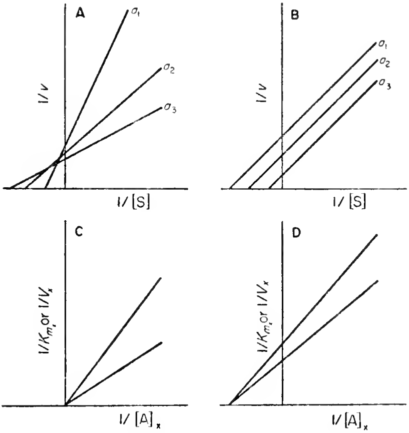

FIG. 4. Types of plots obtained for various mechanisms of flavin-linked dehydrogenases (see text).

Plots **A** *and* **B.** Plots of $1/v$ against $1/[S]$ for varying acceptor concentrations [A]. Curves (a_1, a_2 and a_3) are shown for conditions $[A]_1 < [A]_2 < [A]_3$.
Plot **A** is for mechanisms *I* and *II*.
Plot **B** is for mechanism *III*.
Plots **C** *and* **D.** Plots of $1/K_{m_x}$ or $1/V_x$ against $1/[A]_x$, where $[A]_x$ is any acceptor concentration.
Plot **C** is for mechanism *I*.
Plot **D** is for mechanism *III*.

Here, $$v = \frac{V}{1 + \frac{K}{[A]} + \frac{K_m}{[S]}}, \quad V_x = \frac{V}{1 + \frac{K}{[A]_x}} \quad \text{and} \quad K_{m_x} = \frac{K_m}{1 + \frac{K}{[A]_x}}$$

In this case, K_{m_x}/V_x is constant, and plots of $1/V_x$ or $1/K_{m_x}$ against $1/[A]_x$ are linear, with intercepts on the vertical axis of $1/V$ or $1/K_m$, and on the horizontal axis of $1/K$.

From the results presented by Slater (1955), it appears that most flavoproteins conform to either mechanism *I* or *II*. The few exceptions (such as notatin and DPNH-cytochrome *c*-reductase of heart muscle) may be explained in terms of the relative magnitudes of the rate constants involved in V_x and K_{m_x} so that the plot of K_{m_x} against V_x passes through the origin (see Appendix). Figure 4 shows how the three mechanisms may be differentiated.

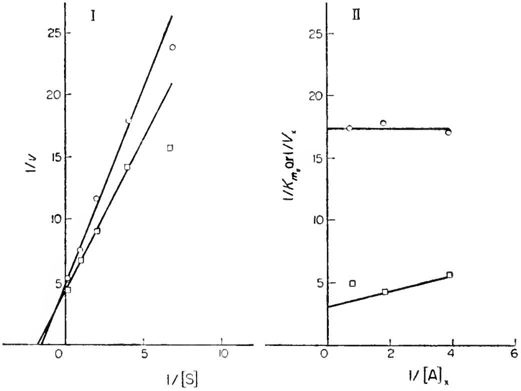

FIG. 5. Lactate-ferricyanide reductase activity of about 2×10^{-8} M crystalline cytochrome b_2 in 0·03 M sodium pyrophosphate–HCl buffer at pH 8·0 and 30°C. Units are as follows: v and V_x, ΔE_{420}mμ/min; [S], L(+)-lactate, mmoles/l.; $[A]_x$, potassium ferricyanide, mmoles/l.

Plot I. Curves for 0·255 mM ferricyanide (—O—) and 0·720 mM ferricyanide (—□—). Compare Fig. 4, Plots **A** and **B**.

Plot II. Curves for $1/K_{m_x}$ (—O—) and $1/V_x$ (—□—) against $1/[A]_x$. (The lowest value of $1/V_x$ was not considered in fitting the curve, as the rate was clearly inhibited at high ferricyanide concentrations). Compare Fig. 4, Plots **C** and **D**.

As shown in Fig. 5, results obtained for the lactate-ferricyanide reductase activity of cytochrome b_2 appear to conform to mechanism *I* or *II*, although the latter seems more probable. However, as shown by Fig. 6, the lactate-cytochrome *c* reductase activity clearly conforms with mechanism *III*.

From the considerations presented above, we interpret the results in the following way.

The enzyme-lactate complex is one in which the greatest part of the two electrons of the lactate are donated to the flavin moiety. The oxidation of this complex by ferricyanide takes place through a ternary complex. This is consistent with the general behaviour observed for flavoprotein enzymes.

The reaction with cytochrome *c* requires an intermediate reduced form of the enzyme. It is postulated that the protohaem moiety of cytochrome b_2 reacts at the same site as the ferricyanide, oxidizing the lactate-flavin complex, and thus forms the intermediate reduced form of the enzyme. This is in turn oxidized by the cytochrome *c*.

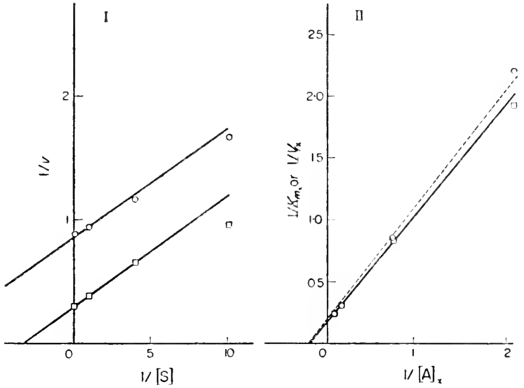

FIG. 6. Lactate-cytochrome *c* reductase activity about 2 × 10^{-8} M crystalline cytochrome b_2 in 0·03 M sodium pyrophosphate–HCl buffer at pH 8·0 and 30°C. Heart-muscle cytochrome *c* purified by resin chromatography was used.

Units are as follows: v and V_x, ΔE_{550} mμ/min; [S], L(+)-lactate, mmoles/l.; $[A]_x$, heart-muscle cytochrome *c*, mmoles/l.

Plot I. Curves for 15 μM cytochrome *c* (—O—) and 82 μM cytochrome *c* (—□—). Compare Fig. 4, Plots **A** and **B**.

Plot. II. Curves for $1/K_{m_x}$ (—□—) and for $1/V_x$ (—O—).

These observations raise the question as to whether the reactions between the flavin and haem groups of the yeast lactate dehydrogenase system are truly intramolecular, viz. between two prosthetic groups of the one enzyme, or intermolecular, viz. between prosthetic groups of two different proteins. The former view is supported by the extensive purification studies, physical studies, and degradation studies of the enzyme as discussed in the foregoing section. Moreover, Chance, Klingenberg and Boeri (1956) titrated cytochrome b_2 with lactate and observed that the reduction of the flavin and haem portions is virtually simultaneous. This implies a close juxtaposition of the flavin and haem groups.

The results of Yamashita *et al.* (1958) show that the flavin-free haemoprotein derivative of cytochrome b_2 is reduced by active cytochrome b_2 (yeast lactate dehydrogenase) at only about 0·02 times the rate of cytochrome *c*. Assuming that the haemoprotein derivative of cytochrome b_2 is undenatured, it is clear that an inter-molecular reaction between flavoprotein and this haemoprotein could not account for the observed rate of reduction of cytochrome *c*.

Kinetic studies with DCIP as acceptor are being carried out but only preliminary results are available as yet. However, comparison with the succinate dehydrogenase system, as discussed by Morton (1955a) suggests that dyes interact with cytochrome b_2 at the haem, rather than at the flavin group, as shown in Fig. 1. Recent results of Horio, Yamashita and Okunuki (1959) are consistent with this formulation.

SUMMARY

1. The results presented here show that cytochrome b_2 is a flavohaemoprotein having equimolecular proportions of riboflavin phosphate and of protohaem and having lactate dehydrogenase activity. A deoxyribose polynucleotide associated with the crystalline enzyme has no influence on this enzymic activity. Cytochrome b_2 contains no iron in excess of that in the protohaem group.

2. The weight/mole of haem is 80,000 g.

3. There is considerable specificity of cytochrome b_2 at the substrate-binding site.

4. The linkage between flavin and protein appears to involve hydrogen bonding of the imino group of the isoalloxazine ring to a cysteine residue of the protein.

5. The amino acid composition of cytochrome b_2 is given. Ligands at positions 5 and 6 of the iron atom of the protohaem are provided by amino acid residues of the protein; probably one position at least is occupied by an imidazole group of a histidine residue.

6. An intermediate oxidation state of the cytochrome has been detected spectroscopically. The shift of the absorption bands to longer wavelengths as compared with those of the fully reduced cytochrome suggests interaction between substrate, flavin and haem groups.

7. The change in autoxidizability of cytochrome b_2 on dissociation of the flavin, and other evidence, indicate a close juxtaposition of the flavin and haem groups of the protein.

8. Kinetic evidence supports different mechanisms for the reactions of the enzyme with ferricyanide and with cytochrome *c*. The mechanism of the reaction of cytochrome b_2 with various acceptors is discussed.

9. The results may be of significance for understanding hydrogen transport during terminal respiration in mitochondria.

Appendix

The mechanisms given here have the following steady-state rate equations

I. (cf. p. 516)

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \tag{1}$$

$$ES + A \xrightarrow{k_3} E + P + AH_2 \tag{2}$$

(where A and AH_2 represent the oxidized and reduced forms of a two-electron acceptor).

$$v = \frac{k_3[E][A]}{1 + \dfrac{k_2 + k_3[A]}{k_1[S]}}, \qquad V = k_3[E][A] \qquad K_m = \frac{k_2 + k_3[A]}{k_1}$$

Thus, only where $k_2 \ll k_3[A]$ does K_{m_x}/V_x remain constant.

For a one-electron acceptor, the mechanism becomes:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \tag{1}$$

$$ES + A \xrightarrow{k_3} E'' + P + HA \tag{3}$$

$$E'' + A \xrightarrow{k_5} E + HA \tag{4}$$

or

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \tag{1}$$

$$ES + A \xrightarrow{k_3} (ES)' + HA \tag{5}$$

$$(ES)' + A \xrightarrow{k_5} E + P + HA \tag{6}$$

$$v = \frac{k_3[E][A]}{1 + \dfrac{k_3}{k_5} + \dfrac{k_2 + k_3[A]}{k_1[S]}}$$

In this case also, both K_{m_x} and V_x have no finite limit values.

II. (cf. p. 516)

(*a*) *See below.*

(*b*)

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$$

$$ES + A \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ESA$$

$$ESA \xrightarrow{k_5} E + P + H_2A$$

$$v = \frac{k_5[E]}{1 + \dfrac{k_4 + k_5}{k_3[A]} + \dfrac{k_2(k_4 + k_5)}{k_1 k_3[A][S]} + \dfrac{k_5}{k_1[S]}}$$

A similar rate equation is obtained for mechanism *II* (*a*) (cf. p. 516). The corresponding rate equations for one-electron acceptors have the same general form, but require certain restrictions as to rate-limiting steps for their derivation.

III. (cf. p. 517)

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \tag{1}$$

$$\mathrm{ES} \xrightarrow{k_3} \mathrm{E}' + \mathrm{P} \tag{10}$$

$$\mathrm{E}' + \mathrm{A} \xrightarrow{k_5} \mathrm{E} + \mathrm{H_2A} \tag{11}$$

$$v = \frac{k_3[\mathrm{E}]}{1 + \dfrac{k_3}{k_5[\mathrm{A}]} + \dfrac{k_2 + k_3}{k_1[\mathrm{S}]}} \qquad V_x = \frac{k_3[\mathrm{E}]}{1 + \dfrac{k_3}{k_5[\mathrm{A}]_x}} \quad \text{and} \quad K_{m_x} = \frac{\dfrac{k_2 + k_3}{k_1}}{1 + \dfrac{k_3}{k_5[\mathrm{A}]_x}}$$

For a one-electron acceptor:

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \tag{1}$$

$$\mathrm{ES} \xrightarrow{k_3} \mathrm{E}'' + \mathrm{P} \tag{12}$$

$$\mathrm{E}'' + \mathrm{A} \xrightarrow{k_5} \mathrm{E}' + \mathrm{HA} \tag{13}$$

$$\mathrm{E}' + \mathrm{A} \xrightarrow{k_7} \mathrm{E} + \mathrm{HA} \tag{14}$$

$$v = \frac{k_3[\mathrm{E}]}{1 + \dfrac{k_3(k_5 + k_7)}{k_5 k_7[\mathrm{A}]} + \dfrac{k_2 + k_3}{k_1[\mathrm{S}]}}$$

As indicated above, some consideration has been given to the cases for one-electron and two-electron acceptors, and it has been found that the types of mechanisms given hold for either case, provided some minor modifications of the detailed mechanisms are made. In the early part of the reaction, the flavin moiety is probably alternately oxidized and reduced between the fully reduced and *semi*quinone states, without ever reaching the fully oxidized state. This assumption results in some simplification of the postulated mechanisms.

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CONDITIONS FOR THE AUTOXIDATION OF FLAVOCYTOCHROME b_2

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INTRODUCTION

Flavocytochrome b_2 is the L(+)-lactate dehydrogenase of aerobic yeast. Several acceptors can be used for the oxidation of lactate by this enzyme; most of these acceptors are artificial. Ferricytochrome *c* (which is also present in aerobic yeast) is probably the physiological acceptor. Oxygen is also an acceptor, the flavocytochrome b_2 having been described as autoxidizable by Appleby and Morton (1954), by Boeri and Tosi (1956) and as 'very slightly autoxidizable' by Morton (1958).

It is the purpose of the present investigation to gain information on how efficient an electron acceptor oxygen can be for flavocytochrome b_2. This information can tell us how likely is a direct by-pass of the usual cytochrome route. Furthermore, a study of the conditions needed for the autoxidation of a cytochrome can give useful general information, as the reasons making certain cytochromes (like cytochrome a_3 and b_1) readily autoxidizable and others (like cytochrome *c*) only autoxidizable under extreme conditions are still poorly understood.

METHODS

Extraction of Flavocytochrome b$_2$

Compressed aerobic bakers' yeast was obtained from the factory and dried. Lots of 3 kg of dried yeast were used each time. The yeast was ground, suspended in 3 × its weight 0·1 M K_2HPO_4, blended in a Waring blendor, and autolyzed for 45 min at 37°C. After centrifugation, the supernatant was brought to pH 5·6 with 0·5 M lactic acid and heated for 15 min at 52°C. After cooling and centrifugation, the supernatant was diluted with an equal amount of distilled water and brought to pH 5·4 with lactic acid. Calcium phosphate gel (45 mg dry weight/ml) was added in a ratio 1:6 and the mixture was slowly agitated for 30 min. The centrifuged gel was treated with a solution which was 0·1 M in K_2HPO_4, 0·02 M in sodium lactate and 20%

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* Professor Enzo Boeri died in October, 1960 (Editors).

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saturated with ammonium sulphate. This solution caused the elution of the enzyme. The eluted material was treated with solid ammonium sulphate to a final saturation of 90% and kept at 4°C for at least overnight.

The precipitate was centrifuged and washed with 60% saturated ammonium sulphate, and finally dialyzed for 3 hr in the cold against 0·05 M sodium lactate under the exclusion of the air. The solution was then treated with cold acetone, which was slowly added under agitation until the cytochrome spectrum disappeared from the supernatant. The excess of acetone was removed by centrifugation and vacuum suction. The precipitate was suspended in 0·01 M lactate and dialysed as before. Insoluble material was discarded. The enzyme solution was brought over a column of DEAE-cellulose according to Nygaard (1958) and purified according to the same author. The eluted material was finally adsorbed onto and eluted from calcium phosphate gel as before, precipitated with 70% saturation of ammonium sulphate and dialysed (as before) to remove the salt.

In summary, this procedure contains steps from the purification according to Bach, Dixon and Zerfas (1946), Appleby and Morton (1954, 1959) and Nygaard (1958). The yield is about 0·1 micromole flavocytochrome b_2/kg of dried yeast.

We were unable to obtain the crystalline preparation described by Appleby and Morton (1954, 1959; but see Note, p. 533). Our best preparation had a content of one flavin monophosphate and one haematin residue for each 140,000 g of protein. The turnover number was 16,000 moles ferricyanide reduced by one mole of enzyme/min in the presence of excess lactate. This corresponds to 6,900 Morton units/mg of protein.

Other Techniques

Oxygen consumption was measured in a Warburg manometric apparatus at 37°C.

L(+)-Lactate determinations were performed with an enzymic method, by measuring the amount of ferricyanide reduced in the presence of excess flavocytochrome b_2. The lactate used was 90% in the L(+) form and 10% in the D(−) form.

Pyruvate determinations were performed according to the method of Friedmann and Haugen (1943).

Catalase was extracted from horse liver according to Sarkar and Sumner (1955).

RESULTS

Effect of the Enzyme Concentration

Figure 1 shows the effect of the concentration of flavocytochrome b_2 on the oxygen consumption for the oxidation of L(+)-lactate as catalysed by this enzyme. The effect is linear.

Effect of the Concentration of Lactate

L(+)-Lactate is the specific substrate. There was no oxygen consumption without addition of lactate. Equally, no oxygen consumption was found in the absence of the enzyme. It is known that when ferricytochrome *c* or ferricyanide is used as the oxidant, the velocity increases with increasing lactate concentration up to a saturation value, these curves allowing calculation of the Michaelis constant. When instead oxygen was the oxidant, a clear inhibiting effect of the substrate was seen, as it appears from Fig. 2.

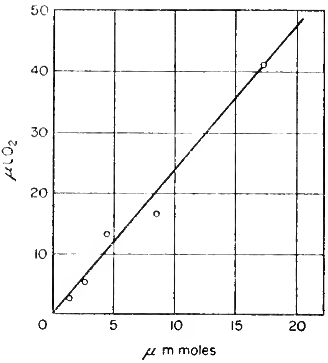

FIG. 1. The effect of the concentration of flavocytochrome b_2 on the oxidation of lactate by oxygen. Abscissa: mμmoles of enzyme in the Warburg vessel. Ordinate: μl. of oxygen consumed in 15 min at 37°C, 120 strokes/min. Phosphate buffer, 0·01 M, pH 6. Lactate concentration 0·002 M. Enzyme added at time zero from the side arm. Gas phase: oxygen.

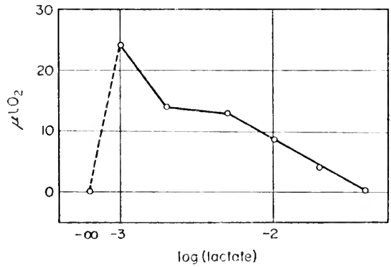

FIG. 2. The effect of the concentration of L(+)-lactate. Abscissa: logarithm of the molarity of lactate. Ordinate: oxygen consumption in 25 min. Enzyme added: 3·75 mμmoles. Total volume: 3·1 ml. Other conditions as in Fig. 1.

Effect of the Oxygen Pressure

Figure 3 shows the effect of the oxygen pressure on the reaction rate. It appears that the reaction rate is larger when the oxygen pressure is increased.

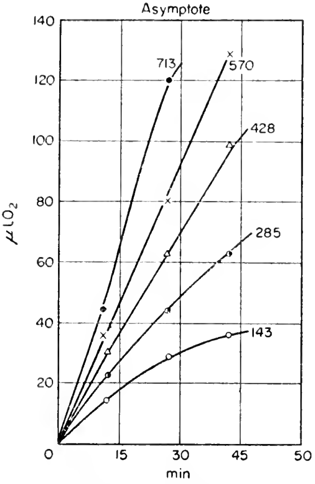

FIG. 3. The effect of the oxygen pressure on the rate of the reaction. Abscissa: time in min. Ordinate: oxygen consumption. The number near each tracing shows the oxygen pressure in mm Hg. Enzyme added: 17 mμmoles. Other conditions as in Fig. 1. The line marked 'asymptote' corresponds to the theoretical consumption for the oxidation of lactate present.

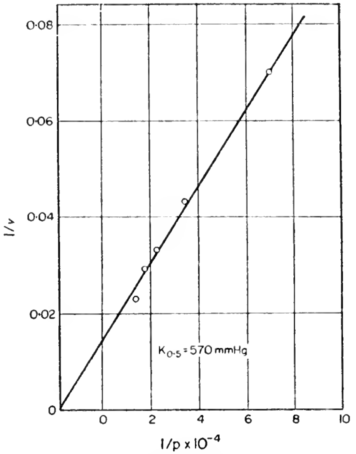

FIG. 4. Lineweaver and Burk plot of the experimental data of Fig. 3. Abscissa: the reciprocal of the oxygen pressure (in mm Hg). Ordinate: the reciprocal of the oxygen consumption (μl. in 15 min).

Figure 4 is a plot according to Lineweaver and Burk. The oxygen pressure at which, under the conditions of the experiment, the rate is half-maximal, is, according to this figure, 570 mm Hg.

Effect of pH

Figure 5 shows the effect of pH on the oxidation rate. The rate is maximal around neutrality. It decreases clearly when the solution becomes more acid.

The Effect of Ionic Strength

Figures 6 and 7 show the effect of the ionic strength. The ionic strength was calculated according to the equation

$$\omega = \tfrac{1}{2}\,\Sigma m_i z_i^2$$

where m_i is the molar concentration of each ion and z_i is its valence. The ionic strength was arranged by adding increasing amounts of sodium chloride to the solution. It appears that a definite ionic strength is needed for the reaction. At high ionic strength the reaction rate decreases slowly again.

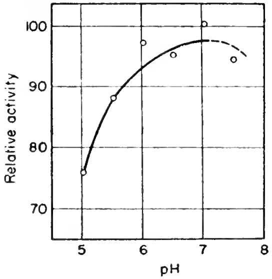

FIG. 5. The effect of pH on the rate of the reaction. Abscissa: time in minutes. Ordinate: activity in fractions of the maximal (at pH 7). Phosphate buffer: 0·01 M. Other conditions are as in Fig. 1.

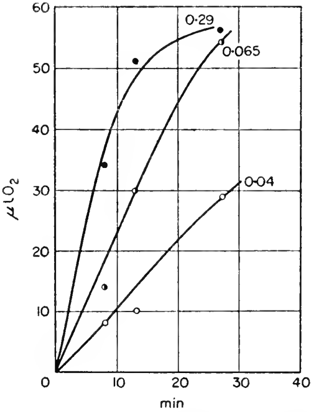

FIG. 6. The effect of the ionic strength on the reaction rate. Abscissa: time in min. Ordinate: oxygen consumption. The number near each tracing indicates the ionic strength. Enzyme added: 14 mμmoles. Other conditions as in Fig. 1.

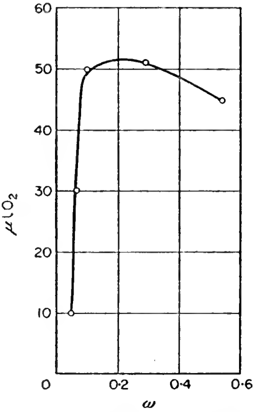

FIG. 7. The effect of the ionic strength on the reaction rate. Abscissa: ionic strength. Ordinate: oxygen consumption in 13 min. Enzyme added: 14 mμmoles. Other conditions as in Fig. 1.

The Effect of the Addition of Catalase

In order to ascertain whether the oxidation of lactate occurs through formation of hydrogen peroxide, catalase was added. As it appears from

Fig. 8, the reaction proceeds at the same rate both in the absence and in the presence of catalase. The enzyme itself was devoid of catalase activity.

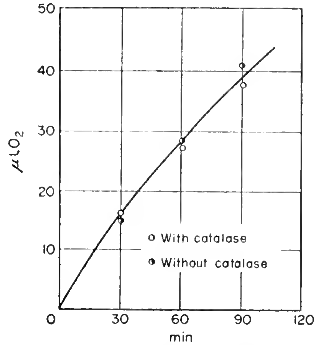

FIG. 8. The effect of the addition of catalase. Abscissa: time in min. Ordinate: oxygen consumption. Flavocytochrome b_2 added: 4·25 mμmoles. Catalase added: 56 mμmoles.

The Stoichiometry of the Reaction

Oxidation of lactate by molecular oxygen could proceed either through the reaction

$$CH_3CHOHCOO^- + O_2 \rightarrow CH_3COCOO^- + H_2O_2 \qquad (1)$$

or through the reaction

$$2CH_3CHOHCOO^- + O_2 \rightarrow 2CH_3COCOO^- + 2H_2O \qquad (2)$$

TABLE 1. OXIDATION OF LACTATE BY CYTOCHROME b_2 WITH OXYGEN

Determination of the stoichiometry of the reaction. Reaction time: 60 min. Enzyme added: 30 mμmoles

Experiment No.	*a* O$_2$ consumed (μmoles)	*b* Lactate oxidized (μmoles)	*a*/*b*
1	4·0	8·6	0·47
2	4·9	11·2	0·44
3	5·2	11·2	0·47
		pyruvate formed (μmoles)	
4	1·2	2·6	0·46
5	2·1	4·0	0·52
6	1·8	4·0	0·45

The latter reaction is followed in this instance, as it appears from Table 1. One mole of oxygen is consumed for two molecules of lactate disappearing, and for two molecules of pyruvate formed.

The Effect of Metals

Certain metals were found to affect the autoxidation rate. Copper and iron were found to possess the most evident influence. Figure 9 shows their action. Copper inhibits strongly the autoxidation. Ferrous and ferric iron instead promote it. Ferrous ions have a more marked effect.

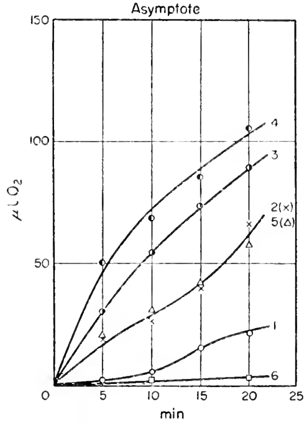

FIG. 9. The effect of the addition of metals on the autoxidation rate. Abscissa: time in minutes. Ordinate: μl. of oxygen consumed. No. 1: no addition. No. 2: in the presence of 0·1 mM $FeCl_3$. No. 3: in the presence of 0·1 mM $FeSO_4$. No. 4: in the presence of 0·1 mM $FeSO_4$ and 0·1 mM *o*-phenanthroline. No. 5: in the presence of 0·1 mM $FeSO_4$ and 0·3 mM *o*-phenanthroline. No. 6: in the presence of 0·1 mM $CuSO_4$. Enzyme added: 17mμmoles. Gas phase: oxygen. The line marked 'asymptote' corresponds to the theoretical consumption for the autoxidation of the lactate present. pH 6 was obtained by addition of diluted acetic acid and sodium hydroxide, and controlled before and after the incubation at 37°C.

In view of this effect, *o*-phenanthroline was tested. Strangely enough, it was found that the combination of equimolar amounts of *o*-phenanthroline and ferrous ions had a stimulating effect which was even higher than that of ferrous ions alone. When, instead, *o*-phenanthroline was added in a ratio 3:1 to the amount of ferrous iron, the effect on the autoxidation rate was lower than with ferrous ions alone, but it was still evident.

When now the effect of the substrate concentration (lactate) was reinvestigated in the presence of excess of ferrous iron, the inhibiting effect of high lactate concentration was absent. Figure 10 enables calculation of the lactate concentration giving half-maximal rate (in the presence of excess ferrous ions); it appears to be 0·5 mM.

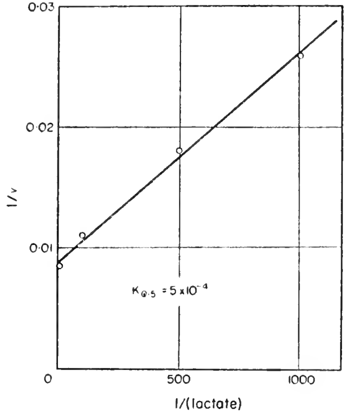

FIG. 10. The effect of the concentration of the L(+)-lactate in the presence of mM $FeSO_4$. Abscissa: reciprocal of the molarity of lactate. Ordinate: reciprocal of the oxygen consumption (μl.) in 10 min at pH 6 and 37°C.

DISCUSSION

Flavocytochrome b_2 preparations catalyse reaction (2) above.

The reaction proceeds faster with more enzyme and with more oxygen present. The optimal pH is around neutrality. At pH values lower than 6 the reaction slows down. This behaviour is similar to what happens with other oxidants (ferricytochrome *c*, ferricyanide, phenazine methosulphate) and is similarly explained by the presence of a group on the enzyme dissociating with a p*K* around 5·65.

With proper lactate, salt, hydrogen ion and oxygen concentrations the turnover number for oxygen appears to be 27 moles oxygen consumed/mole of enzyme/min as calculated from the values of Fig. 6. Since oxygen accepts four electrons/molecule, the turnover number for oxygen as oxidant is of 108 equivalents/mole of enzyme/min, in comparison with 16,000 for ferricyanide and ferricytochrome *c*. Therefore, oxygen under the best conditions is only about 0·7% as efficient as ferricyanide. In terms of $-Q_{O_2}$, the preparation has a value of 260, which proves rather low in comparison with other oxidases.

Lactate, which is the substrate, inhibits the autoxidation. This means that there is no oxygen consumption without lactate, but the oxygen consumption is blocked by increasing the lactate concentration. As metal ions increase the oxygen consumption, the obvious explanation seems that lactate binds the metal ions and therefore inhibits the autoxidation.

Figures 9 and 10 show that this explanation is correct. Among metals tested, iron was found the most active catalyser. Less active were cobalt ($CoCl_2$), manganese ($MnCl_2$) and molybdenum (Na_2MoO_4).

Copper ($CuSO_4$) strongly inhibited the autoxidation.

The effects of *o*-phenanthroline were difficult to interpret. It was expected that *o*-phenanthroline, as a complexing agent for bivalent iron, would remove the effect of this metal. However, it was found that when *o*-phenanthroline and ferrous ions were added, the effect depended on the ratio of the two reagents. With a ratio of 1 : 1, the catalytic effect on the autoxidation is even higher than with ferrous ions alone. It is known that *o*-phenanthroline is a tridentate chelating agent and binds ferrous ions up to formation of the complex Fe^{II}-(*o*-phenanthroline)$_3$. When this complex was tested, it was still found to be catalytically active on the autoxidation rate, although less active than ferrous iron alone. Actually, this fact appears less strange when we think that a similar compound, ferricyanide, which also has six valences of iron blocked in a diamagnetic compound, is very accessible to flavocytochrome b_2, being the acceptor of choice for testing its activity (Appleby and Morton, 1954).

Finally, it must be remembered that high amounts of non-haematin iron accompany the enzyme during its purification, only the last step removing the last traces of it. This seems the reason why the unpurified enzyme appears much more autoxidizable than purified preparations. Our present preparations have only traces of non-haematin iron.

Factors which favour the autoxidation (high oxygen and metal and low lactate concentrations) tend to inactivate the enzyme. We have computed from activity analysis that the enzyme has lost about half its activity when it has cycled 750 times with oxygen as the acceptor. The decay is infinitely less when ferricytochrome or ferricyanide are the acceptors.

According to Morton (1958), the fact that removal of heavy metals and oxygen protects the enzyme is in favour of a bond between —SH groups of certain of the cysteine residues of the protein and the imino group of the riboflavine phosphate. The present results are in favour of such an explanation.

The effect of ionic strength should be compared to the effect shown when other oxidants are used. When ferricyanide is the oxidant, there is no effect due to the ionic strength. When cytochrome *c* is the oxidant, high ionic strength inhibits the reaction. With oxygen, instead, there is an optimal ionic strength.

In conclusion, as noted by Morton (1958), flavocytochrome b_2 is a very slightly autoxidizable substance. The complete inhibition of autoxidation exerted by lactate and the effect of metals place flavocytochrome b_2 among substances like cysteine and ascorbic acid, which are only autoxidizable in the presence of metals.

SUMMARY

The oxidation of lactate to pyruvate, catalysed by flavocytochrome b_2, was studied using oxygen as the electron acceptor. The effects of the concentration of enzyme, lactate, oxygen, hydrogen ions, and of ionic strength were studied.

Under optimal conditions, 27 moles of oxygen were reduced/mole of enzyme/min. One molecule of oxygen was consumed for each two molecules of lactate dehydrogenated to pyruvate.

Ferrous and ferric ions actively catalyse the autoxidation of flavocytochrome b_2.

NOTE

Since preparation of this paper, crystalline flavocytochrome b_2 has been obtained according to the procedure described by Appleby and Morton (1954, 1959).

Acknowledgements

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KINETIC STUDIES ON THE ACTION OF YEAST LACTATE DEHYDROGENASE

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Since Bernheim (1938) studied the properties of lactate dehydrogenase using a crude cell free extract obtained from bakers' yeast, many investigators have worked on the properties of this enzyme. Using an enzyme preparation of sufficient purity, Dixon and his co-workers (Dixon and Zerfas, 1939; Bach, Dixon and Zerfas, 1942, 1946) observed that cytochrome b_2 was always present in the enzyme system. At that time, however, they could not decide whether or not the protohaem present was a prosthetic group of lactate dehydrogenase itself. Five years ago, Appleby and Morton (1954, 1959) succeeded in crystallizing this enzyme and established that the enzyme contains two different prosthetic groups, namely protohaem and flavin mononucleotide. The fact that the flavin mononucleotide forms an essential part of the enzyme itself, was also ascertained by Boeri and his co-workers (Boeri, Cutolo, Luzzati and Tosi, 1955; Boeri and Tosi, 1956). Based on their further observations, they inferred that in addition to the two different prosthetic groups mentioned above, eight iron ions bound to the enzyme protein seemed to participate as the essential factors in the enzymic action.

The investigation to be reported here was undertaken to obtain further information concerning the mechanism of lactate dehydrogenase. Our main interest was to elucidate in what manner the protohaem and flavin mononucleotide behave as electron carriers in the mechanism of the enzymic action.

EXPERIMENTAL

Bakers' yeast supplied from the Oriental Yeast Co. was used as the source of the enzyme. The procedures of enzyme purification were almost the same as those described by Bach *et al.* (1946). After the cells were plasmolysed by the addition of ethyl acetate, they were subjected to extraction with phosphate buffer. The purification was performed by selective adsorption with calcium phosphate gel followed by fractional precipitation with ammonium sulphate. The enzyme samples used throughout these experiments were spectrophotometrically free from cytochrome *c*.

In the kinetic experiments, the rates of reduction of dyes were measured under anaerobic conditions with a photoelectric colorimeter using Thunberg tubes, whereas those of cytochrome *c* and ferricyanide were determined spectrophotometrically. Prior to the experiments, the concentrations of dyes were determined by careful titration with a standardized dithionite solution under anaerobic condition.

In most of the experiments racemic lactate was used as the substrate. The concentrations of substrate described in the data were those of L-lactate contained in the reaction mixtures, since it has been confirmed that D-lactate did not show any effect on the enzymic reaction, as mentioned by Boeri *et al.* (1955).

RESULTS AND DISCUSSION

Properties of the Enzyme

The spectrum of the enzyme, reduced by the addition of lactate, showed maxima at 557, 528 and 423 mμ; the Soret maximum of the oxidized form was at 412 mμ. These results were in good agreement with those reported by other authors (Appleby and Morton, 1954, 1959; Boeri *et al.*, 1955).

The spectrum of flavin mononucleotide attached to the enzyme protein was not observed by direct spectrophotometry, since it was largely covered by that of cytochrome b_2. Using a tonometer-type chamber reported by Nakamura (1958), we could measure the spectrum of the former compound by the following procedures. After the air in the chamber was evacuated and replaced by nitrogen gas, a small amount of lactate (final concentration about 50 μM) was added to the buffered enzyme solution placed in the main chamber. The complete reduction of cytochrome b_2 was checked spectrophotometrically, and then the enzyme solution was titrated carefully with an oxygen-free methylene blue solution. When the blue colour of the oxidized form of the dye appeared slightly, the titration was stopped and the mixture was allowed to stand for several minutes. Figure 1 shows the difference spectrum between the enzyme solution thus treated and the reduced one (in which both the protohaem and flavin mononucleotide had been completely reduced). The spectrum, which showed two maxima at 385 and 445 mμ, seemed to be due to the oxidized form of flavin mononucleotide. Conceivably, the flavin mononucleotide moiety of the enzyme was oxidized by the treatment described above, while the protohaem moiety remained in a reduced state. Assuming that the millimolar extinction coefficient of flavin mononucleotide attached to the enzyme protein was the same as that of the free molecule, its concentration in the mixture was estimated to be 16·4 μM from the value of $\varepsilon_{\text{ox}} - \varepsilon_{\text{red}} = 9{\cdot}0\ \text{cm}^{-1}\ \text{mM}^{-1}$. Since the concentration of cytochrome b_2 was found to be 18·6 μM, it was deduced that the molecular ratio of flavin mononucleotide to protohaem present in one molecule of enzyme is 1:1 as reported by Appleby and Morton (1954).

The effect of riboflavin upon the enzymic reaction was investigated as follows. After the enzyme solutions were mixed with various concentrations of riboflavin, the rate of reduction of thionine was measured (by the extinction at 660 mμ) in the presence of an excess of substrate. As may be seen from the results presented in Fig. 2, the inhibition caused by riboflavin increased with time as the reaction proceeded. It is worth mentioning that no inhibitory effects of riboflavin could be observed when the rate of reduction of cytochrome b_2 was measured under similar conditions. The data presented in Fig. 2 seem to suggest that the flavin mononucleotide bound to the enzyme protein became mobilized and exchanged with the added riboflavin when the enzyme was put into its function.

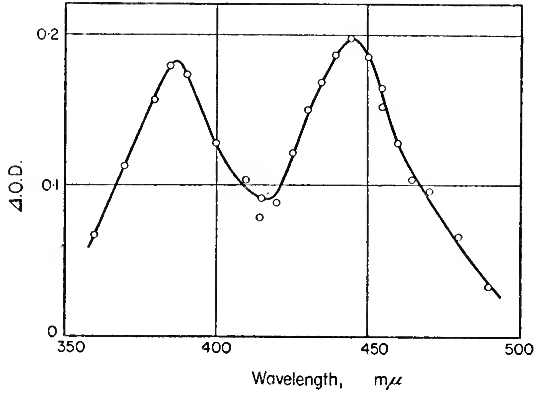

FIG. 1. Difference spectrum of flavin mononucleotide attached to the enzyme protein.

It was further checked whether or not the flavin mononucleotide attached to the enzyme could be removed on the addition of an excess of riboflavin. After the enzyme solution was mixed with an excess of riboflavin, the mixture was allowed to stand for several hours and then treated with ammonium sulphate solution. The precipitate was washed several times with ammonium sulphate solution. The flavin moiety of the enzyme thus treated was identified as riboflavin by paper chromatography. On adding the substrate to the enzyme thus treated, the protohaem moiety of the enzyme was not reduced; the reduction could be achieved only by treatment with dithionite. The observation made above indicates that the flavin mononucleotide moiety of the enzyme can be replaced by riboflavin leading to the formation of an inactive enzyme complex, and that the flavin mononucleotide attached to the enzyme acts as the direct acceptor of electrons derived from substrate prior to the reduction of cytochrome b_2 as was first assumed by Appleby and Morton (1954).

To elucidate further the situation of electron transfer in the enzymic reaction, spectrophotometric measurement was made of the oxidation-reduction potential of cytochrome b_2. An enzyme solution was placed in a Thunberg tube, the lower part of which was shaped like an optical cell, and which was fitted with a rubber stopper. The side-bulb of the tube contained a solution of reduced toluylene blue (TB) ($E_0' = 0{\cdot}127$ V, pH 7·0) which was to be used as an indicator. After the air in the vessel was completely replaced with nitrogen gas, about half of the toluylene blue was reduced by dithionite using a syringe through the rubber stopper. The mixture of both oxidized and reduced forms of dye was introduced into the buffered enzyme solution. By measurement of the changes of optical density at 557 and 600 mμ, the oxidation-reduction potential of cytochrome b_2 could be determined according to the following equation:

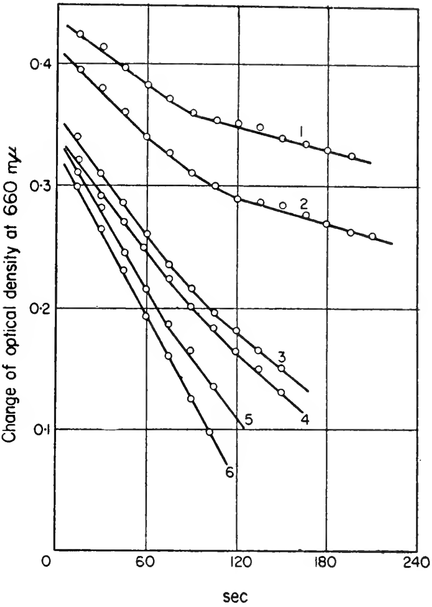

FIG. 2. Inhibitory effect of riboflavin on the enzymic reaction. Concentrations of riboflavin were 200 μM (curve 1), 100 μM (curve 2), 32 μM (curve 3), 10 μM (curve 4), 3·2 μM (curve 5), respectively. Curve 6 represents the control. Experiments were carried out at 25°C and pH 7·2.

$$E_0' \text{ (cyt } b_2) = E_0'(\text{TB}) + 0{\cdot}03 \log \frac{[\text{TB}]}{[\text{leuco TB}]} - 0{\cdot}06 \log \frac{[\text{cyt } b_2^{+++}]}{[\text{cyt } b_2^{++}]}$$

where E_0' (cyt b_2) is the oxidation-reduction potential of cytochrome b_2, and E_0' (TB) is the oxidation-reduction potential of toluylene blue.

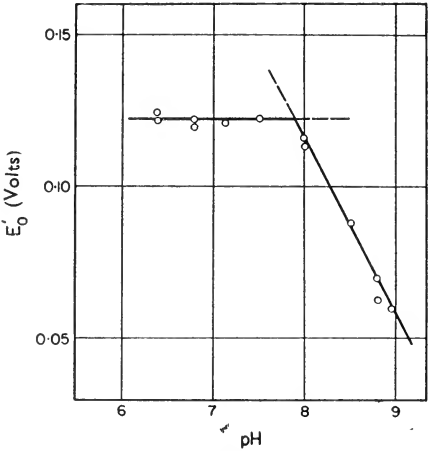

FIG. 3. Values of oxidation-reduction potential of cytochrome b_2 at various pH values.

Figure 3 shows the relationships between the oxidation-reduction potential of cytochrome b_2 and the pH of the medium. The oxidation-reduction potential of cytochrome b_2 thus obtained was + 0·12 V at 25°C and pH 6 to 8.

Kinetics of the Enzymic Reaction

To obtain information as to the mode of action of the enzyme some kinetic investigations were performed. Figure 4 shows the relationships between the rate of reaction and the concentration of enzyme in the presence of methylene blue and ferricyanide as electron acceptors. As may be seen, the rate increased proportionally with the enzyme concentration when both substrate and dye were present in excess. The rates obtained from the slopes of the curves were $1{\cdot}3 \times 10^2 \text{ sec}^{-1}$ (methylene blue) and $1{\cdot}5 \times 10^2 \text{ sec}^{-1}$ (ferricyanide) respectively, the rate being defined as the amount of L-lactate oxidized/unit concentration of enzyme/sec. The effect of dye concentration upon the rate was investigated in the presence of an excess of substrate. Figure 5 shows the relationships between the concentrations of dyes and the rates at 25°C and pH 7·2. At lower concentrations of the dye, the rate was

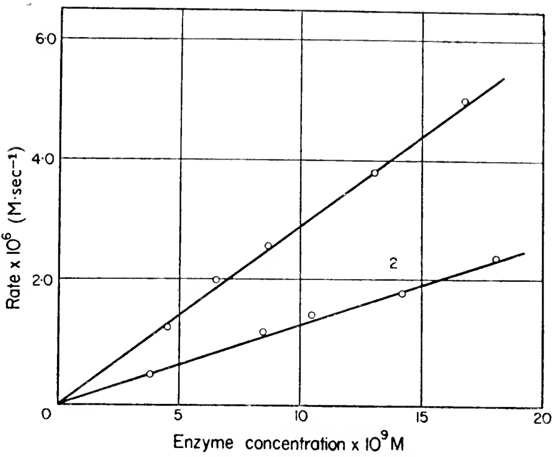

FIG. 4. Relationship between the rate and the concentration of enzyme. Curve 1, ferricyanide; curve 2, methylene blue. Experiments were carried out at 25°C and pH 7·2.

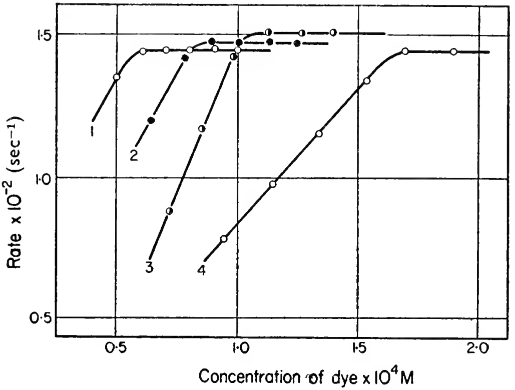

FIG. 5. Relationships between the rates and the concentration of dyes. Curve 1, 2:6-dichlorophenolindophenol; curve 2, thionine; curve 3, methylene blue; curve 4, toluylene blue. Substrate concentration, 10 mM. Experiments were carried out at 25°C and pH 7·2.

a linear function of the dye concentration, while it became saturated at higher dye concentrations. It was further checked that at these saturating concentrations of dyes, dye concentrations decreased linearly with the reaction time.

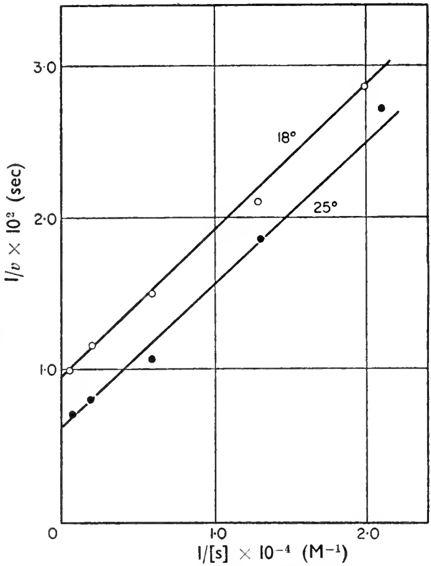

FIG. 6. Relationships between the reciprocal of rate and the reciprocal of substrate concentration. Hydrogen acceptor, thionine. Experiments were carried out at pH 7·2.

Using various kinds of dyes, the dependency of the rate upon the concentration of substrate was investigated according to the method of Lineweaver and Burk (1939).

The data obtained are illustrated in Fig. 6. The maximum rate/unit concentration of the enzyme (V/e) and the Michaelis constant (K_m) are listed in Tables 1 and 2. The values of V/e as well as those of K_m appeared, within the experimental error, to be the same with the different hydrogen acceptors used. It was also found by the double reciprocal method reported by Singer and Kearney (1957) that the same situation holds at lower concentrations of the dye; the result was essentially the same as that given in Table 1.

The oxidation of reduced cytochrome b_2 by ferricyanide and thionine was demonstrated by spectrophotometric observations. In the experiments reproduced in Figs. 7 and 8, the oxidation of cytochrome b_2 proceeded to completion with an excess of ferricyanide (Fig. 7), but only to a certain extent on

TABLE 1. KINETIC DATA FOR YEAST LACTATE DEHYDROGENASE

Values of V/e (maximum rate/unit concentration of enzyme) and of K_m at pH 7·2 and at the temperature indicated, with various acceptors.

Temperature °C	Methylene blue		Thionine		Toluylene blue		2:6-Dichlorophenol indophenol	
	$V/e \times 10^{-2}$	$K_m \times 10^4$	$V/e \times 10^{-2}$	$K_m \times 10^4$	$V/e \times 10^{-2}$	$K_m \times 10^4$	$V/e \times 10^{-2}$	$K_m \times 10^4$
10	0·9	1·0	0·7	0·8	0·71	1·0	0·67	0·8
18	—	—	1·1	1·1	1·4	1·3	1·1	1·0
25	1·35	1·8	1·6	1·3	1·9	1·8	1·8	1·2
33	3·3	2·0	2·7	2·2	2·7	2·0	2·2	1·6

Temperature °C	Ferricyanide		Cytochrome *c*	
	$V/e \times 10^{-2}$	$K_m \times 10^4$	$V/e \times 10^{-2}$	$K_m \times 10^4$
10	—	—	0·72	—
14	0·72	1·0	—	—
20	1·2	1·4	—	—
25	1·5	1·8	1·4	—
31	1·8	2·3	1·8	—

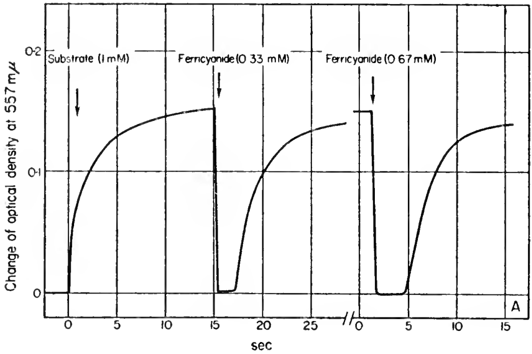

FIG. 7. Oxidation of reduced cytochrome b_2 (change in optical density at 557 mμ) by ferricyanide, at 20°C and pH 7·2.

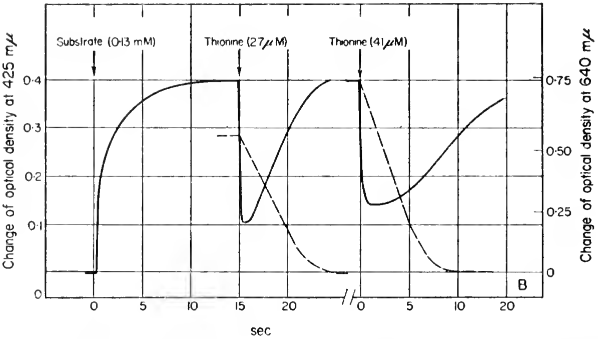

FIG. 8. Oxidation of reduced cytochrome b_2 (change in optical density at 425 mμ) by thionine (solid lines) and reduction of thionine (broken lines; change in optical density at 640 mμ), at 20°C and pH 7·2.

the addition of thionine, which may be attributed to the insufficiency of the dye added. It is interesting to note that the oxidation of reduced cytochrome b_2 can be brought about by dyes such as methylene blue or thionine, with oxidation reduction potentials lower than that of cytochrome b_2.

These data seem to suggest that in the enzymic reaction electron transfer to the dye can occur directly from reduced cytochrome b_2, but not from the reduced flavin mononucleotide.

In order to interpret all the findings described above, the following set of

TABLE 2. FURTHER KINETIC DATA FOR YEAST LACTATE DEHYDROGENASE

Values of V/e, K_m and k_4 obtained by double reciprocal method (see text). Experiments were carried out at 22°C and at pH 7·2.

Acceptor	$V/e \times 10^{-2}$ (sec^{-1})	$K_m \times 10^4$ (M)	$k_4 \times 10^{-6}$ (M^{-1} sec^{-1})
Methylene blue	1·5	2·0	2·7
Thionine	2·7	2·0	8·6
Toluylene blue	1·5	1·6	3·2
2:6-dichlorophenolindophenol	2·0	1·9	4·9
Ferricyanide	1·5	1·5	2·7
Cytochrome *c*	1·4	1·8	4·3

equations may be proposed as representing the mechanism of lactate dehydrogenase.

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightharpoons}} ES \tag{1}$$

$$ES \xrightarrow{k_2} E' + P \tag{2}$$

$$E' \overset{k_3}{\rightleftharpoons} E'' \tag{3}$$

$$E'' + A \xrightarrow{k_4} E + AH_2 \tag{4}$$

where E represents the oxidized form of the enzyme, ES the enzyme-substrate complex, E′ the reduced form of the enzyme, in which the flavin mononucleotide moiety is reduced, E″ another reduced form, in which the protohaem moiety is reduced, S the substrate, A the hydrogen acceptor, P the product and k_{1-4} the rate constants.

Acknowledgement

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VARIOUS FORMS OF YEAST LACTATE DEHYDROGENASE

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Several forms of lactate dehydrogenase have now been isolated from aerobic bakers' yeast (Nygaard, 1958; 1959a, b). The method involved: (1) extraction by the stirring of a yeast suspension with micro glass beads, (2) fractionation with acetone, (3) adsorption on calcium phosphate gel, (4) ammonium sulphate fractionation, and (5) separation on an N,N-diethylaminoethylcellulose (DEAE-cellulose) column. The different forms differed greatly in their affinity for DEAE-cellulose. In the following, three fractions (lactate dehydrogenase (LDH) I, II and III) will be described; they were eluted in that order from the column.

As a reference, the homogeneous and crystalline LDH of Appleby and Morton (1954, 1959) has been used. This enzyme is well defined and well characterized and will be referred to as 'crystalline LDH'. The properties have been described by Appleby and Morton, 1954, 1955, 1959a, b and Appleby, 1957 (see Morton, 1958).

COMPOSITION AND SPECTRAL PROPERTIES OF THE ISOLATED ENZYMES

Lactate Dehydrogenase I (LDH I)

This was eluted with 0·04 M phosphate around pH 6·5. Flavin and haem were eluted as one peak in the ratio 1:2. As in the crystalline enzyme of Appleby and Morton (1954, 1959a, b), the α-band was located at 557 mμ and the Soret band in the reduced and oxidized state was located at 423 mμ and 413 mμ respectively. In contrast to the crystalline enzyme, however, the heights of the reduced and oxidized Soret band were about equal. The extinction coefficient was about 100×10^3 M^{-1} cm^{-2} compared with 240×10^3 for the crystalline enzyme (Appleby and Morton, 1959a, b). Furthermore, all the reduced bands were broader for this fraction. A specific proteolytic attack at the site involved in the binding of the haem may have modified the spectrum. The enzyme gave a normal pyridine protohaemochrome spectrum (Nygaard, 1959b).

Lactate Dehydrogenase II (LHD II)

This was eluted at pH 5·3 with 0·04 M NaCl. The enzyme was not eluted as a distinct protein peak, as the fraction was contaminated by (partly dialysable) polypeptides and also polynucleotides. Proteolytic and nucleolytic degradation took place during the elution procedure. Flavin and haem were eluted as one distinct peak. E_{280}/E_{260} reached a minimum with this peak, thus indicating affinity of the enzyme for the polynucleotides. The purest preparation contained one haem/97,000 g of protein, and was thus as pure as the crystalline enzyme. After dialysis, the enzyme still contained 2–3% of polynucleotides. These were all of the ribose type. The crystalline enzyme contains deoxyribonucleotide (Appleby, 1957; see Morton, 1958). Ribonuclease and deoxyribonuclease did not affect fraction II, just as they did not inactivate the crystalline enzyme (Appleby and Morton, 1955, 1960). Between LDH II and III, cytochrome *c* peroxidase was eluted as a brown zone with one haem/200,000 g of protein.

Some preparations of this material underwent inactivation suddenly (in the course of minutes) when held at 0°C. This was frequently accompanied by the formation of a colourless protein precipitate. This precipitate, which contained much of the nucleotides, may be a part of the enzyme protein. One haem/68,000 g of polypeptides remained dissolved. The inactivation was probably due to proteolytic enzymes, which were present even in the best preparations. Crystalline trypsin and chymotrypsin increased the rate of inactivation.

Inactivation was associated with the appearance of fluorescence (see Appleby and Morton, 1954). The rate of dissociation of the flavin was increased by sodium chloride in the acidic range (around pH 5), thus suggesting that ionic bonds were involved (Theorell and Nygaard, 1954). The dissociation was irreversible. *p*-Chloromercuribenzoate (10^{-4} M) caused fluorescence to appear immediately.

REACTION PROPERTIES

Crude Extracts

When the acetone precipitate (cf. Nygaard, 1958, 1959a, b) (dissolved in water or in phosphate buffer at pH 7) was incubated at 55–57°C for 5 min. or treated with *p*-chloromercuribenzoate (*p*CMB, 3×10^{-3} M), 90% or more of the LDH activity was destroyed. The properties of the remaining enzyme were quite different from those of the original solution. In the first place, the ratio of the reduction of cytochrome *c* to the reduction of ferricyanide and 2:6-dichlorophenolindophenol was high for the stable enzyme. Secondly, the reaction of the stable enzyme with cytochrome *c* was much more affected by changes in the salt concentration. Figure 1 illustrates the reaction properties of a crude preparation which contained 10% of heat-stable enzyme. It is seen from the figure that both the original preparation and the stable enzyme followed zero-order kinetics with cytochrome *c* as acceptor in the region 10^{-5} M to 10^{-4} M when the phosphate concentration was very low (μ 0·01). When the buffer concentration was increased to μ 0·08, however, the activity of the original enzyme was practically unaltered, whereas the stable enzyme in this buffer followed first-order kinetics with respect to cytochrome *c*. The ratio of rates of reduction of cytochrome *c*/ferricyanide/2:6-dichlorophenol-indophenol ('relative activities') was 12:1:1 for the stable enzyme compared with 0·8:1:1 for the original preparation.

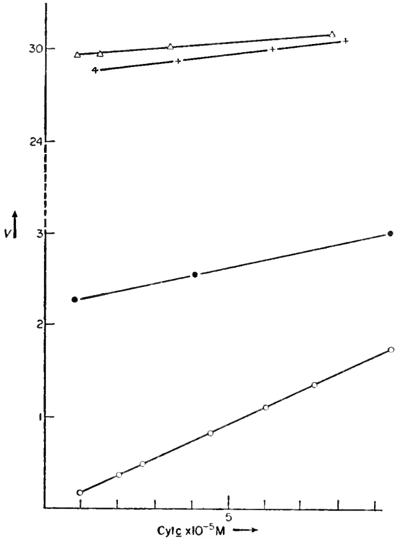

FIG. 1. The effect of the concentration of cytochrome *c* on the rate of oxidation of lactate (DL, 0·02 M) by a crude preparation of LDH and by the heat- and *p*CMB-stable enzyme contained in this preparation; pH 7·1; 0·001 M ethylene-diamine tetra-acetate (EDTA) was added.

△ △ △ Original preparation, in phosphate buffer, μ 0·01
+ + + Original preparation in phosphate buffer, μ 0·08
● ● ● Heat- and *p*CMB-stable enzyme, in phosphate buffer, μ 0·01
○ ○ ○ Heat- and *p*CMB-stable enzyme, in phosphate buffer, μ 0·08

Ordinate: arbitrary velocity units; values were calculated from ΔE at 550 mμ, using $\varepsilon_{\text{mM}} = 18{\cdot}6$.

LDH I

This reduced cytochrome *c*, ferricyanide, and indophenol at substantially the same rate. The turnover number of a preparation was 6,000 moles cytochrome *c* reduced/mole flavin. The enzyme was very labile and half of the activity decomposed in a few hours at 0°C.

LDH II and III

From many preparations only minute amounts of LDH I were obtained. In these preparations LDH II constituted the main part of the LDH activity. The amount of LDH III obtained corresponded roughly to the amount of stable enzyme in the crude preparation.

LDH II was completely inactivated by incubation at 55–57°C for 3 min (pH 7·1) and by 10^{-4} M *p*CMB (0°C, pH 7·1). The reduction of cytochrome *c* by LDH III was unaffected under the same conditions. The reduction of ferricyanide and indophenol, however, was as labile in LDH III as in LDH II. Thus, the relative activities of LDH III could be changed by heat or *p*CMB. The relative activities of LDH II could not be altered in this way, and they remained roughly the same also during inactivation due to ageing, freezing, and proteolytic digestion. This is illustrated in Table 1.

TABLE 1. ABSOLUTE AND RELATIVE ACTIVITIES OF FRESHLY PREPARED AND OF PARTIALLY INACTIVATED LDH II

The activity was determined spectrophotometrically at 20°C. The composition of the medium was: phosphate buffer pH 7·1, ionic strength 0·01; versene 0·001 M; DL-lactate 0·005 M; cytochrome *c* 2 × 10^{-5} M, or ferricyanide to give 10^{-3} M, or 2:6-dichlorophenolindophenol 5 × 10^{-5} M.

Turnover numbers were calculated from ΔE at 550 mμ (cytochrome *c*, ε_{mM} 18·6), 420 mμ (ferricyanide, ε_{mM} 16), and 600 mμ (2:6-dichlorophenolindophenol, ε_{mM} 10). Turnover number is defined as the equivalents of acceptor reduced/mole of flavin/min.

Treatment	Turnover number (μmoles of acceptor reduced/min/mole of flavin or haem at 20°C)			Relative activities
	Cytochrome *c*	Ferricyanide	2:6-dichlorophenol-indophenol	
Freshly prepared	6,300	10,000	6,000	1·0:1·7:1·0
Aged at 6°C for 70 hr	3,300	5,500	3,000	1·1:1·8:1·0
Frozen and thawed	2,000	2,900	2,000	1·0:1·5:1·0
Stored at −10°C for 3 weeks	120	240	120	1·0:2·0:1·0
Trypsin-digested	180	400	—	1·0:2·2

The sensitivity to salt was widely different for LDH II and III. The cytochrome *c*–dependency curve for LDH II and its insensitivity to salts was similar to that of the original crude preparation, and the cytochrome *c*–dependency curve for LDH III and its sensitivity to salt was similar to the stable enzyme of the crude preparation. In general, LDH II followed zero-order kinetics down to a concentration of 10^{-5} M cytochrome *c*, and the rate was the same in phosphate μ 0·01, 0·08 and 0·08 + 0·04 M NaCl. LDH III,

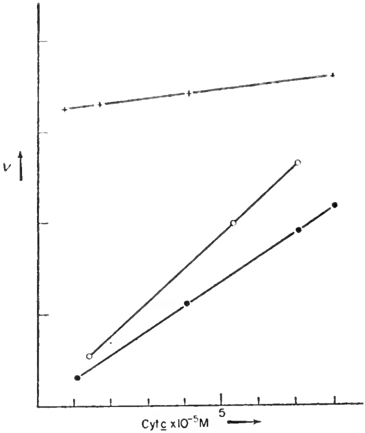

FIG. 2. The effect of the concentration of cytochrome *c* on the rate of oxidation of lactate (DL, 0·02 M) by a preparation of LDH III obtained from the crude preparation described in Fig. 1. All solutions were at pH 7·1, 0·001 M EDTA was added.

+ + + In phosphate buffer, μ 0·01
○ ○ ○ In phosphate buffer, μ 0·08
● ● ● In phosphate buffer, μ 0·08 + 0·04 M NaCl

Ordinate: arbitrary velocity units.

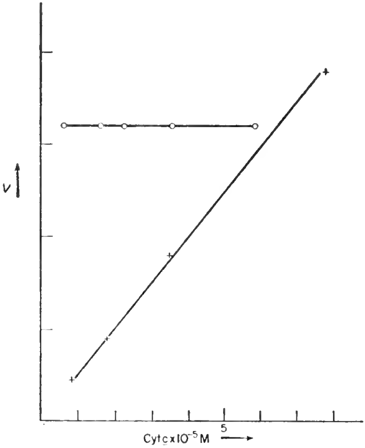

FIG. 3. The effect of the concentration of cytochrome *c* on the rate of oxidation of lactate (DL, 0·02 M) by a preparation of LDH III (the same as for Fig. 2). pH 7·9.

○ ○ ○ In glycylglycine, 0·005 M
× × × In glycylglycine, 0·005 M + 0·04 M NaCl

Ordinate: arbitrary velocity units.

on the other hand, was strongly affected by salts; the affinity for cytochrome *c* was decreased by increased salt concentration. Figure 2 shows the reaction properties of LDH III in three different solutions. In phosphate, μ 0·01, the enzyme followed zero-order kinetics at least down to 10^{-5} M; in phosphate, μ 0·08, the reaction was of first-order up to a concentration of 10^{-4} M cytochrome *c*. The addition of NaCl (0·04 M) decreased the first-order rate constant. Figure 3 illustrates the fact that the same salt dependency exists at pH 7·9. The sodium salts of chloride, sulphate, and acetate had much stronger effects than Na-glycylglycinate. The pH-dependency curves of LDH II and III with cytochrome *c* as acceptor were slightly different. This is shown in Fig. 4.

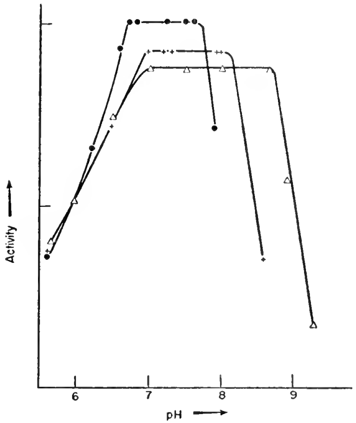

FIG. 4. The rate of the reduction of cytochrome *c* (2×10^{-5} M) by LDH II and III as a function of pH (DL-lactate, 0·02 M).

● ● ● LDH III in 0·002 M phosphate + 0·002 M glycylglycine
+ + + LDH III in phosphate μ 0·08 + 0·002 M glycylglycine + 0·001 M versene
△ △ △ LDH II in phosphate μ 0·008 + 0·001 M versene, below pH 7·7; in glycylglycine, 0·05 M, from pH 7·7 to 8·6; in glycine, 0·05 M, from pH 8·6 to 9·7. Change in the buffer at pH 8·6 and 7·7 did not affect the activity.

Ordinate: arbitrary velocity units.

The reaction of LDH II and III with ferricyanide as acceptor was of zero-order at least down to a concentration of 2×10^{-5} M, and the reaction with indophenol attained half-maximal activity with 3×10^{-5} M. Both enzymes were unaffected by great variations in the salt concentration. The effect of pH on the activity of LDH II and III with ferricyanide as acceptor is shown in Fig. 5. The curves were similar except for the drop in rate on the alkaline side.

The most active preparation of LDH II had relative activities (see earlier) of 1:1:1 and turnover number 15,000 moles of cytochrome *c* reduced/min/mole of flavin or haem. The turnover number of a preparation of LDH III with cytochrome *c* in phosphate μ 0·01, pH 7·1, was 6,000 μmoles of cytochrome *c* reduced/min/mole of flavin. The relative activities of LDH III varied from preparation to preparation. The reduction of ferricyanide and indophenol was usually less than 0·2 times that of cytochrome *c*.

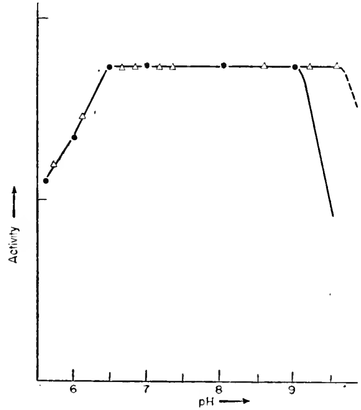

FIG. 5. The rate of the reduction of ferricyanide (5 × 10^{-5} M) by LDH II and III as a function of pH (DL-lactate, 0·02 M).

○ ○ ○ LDH II
△ △ △ LDH III
Buffers used: phosphate μ 0·08 at pH 7·7 and below; glycylglycine, 0·05 M, from pH 7·6 to 8·6; glycine, 0·05 M, from pH 8·6 to 9·7. Change of buffer at pH 8·6 and 7·7 did not affect the activity.

Ordinate: arbitrary velocity units.

LDH II was specific for L-lactic acid, whereas LDH III was specific for D-lactic acid.

DISCUSSION

In a recent note Labeyrie, Slonimski and Naslin (1959) reported that lactate dehydrogenase of anaerobic yeast was specific for D-lactate. This enzyme had previously been shown not to reduce cytochrome *c* (Lindenmayer and Smith, 1957; Slonimski and Tysarowski, 1958; Boeri and Cutolo, 1958). Slonimski and Tysarowski (1958) and Labeyrie *et al.* (1959) proposed that anaerobic yeast lactate dehydrogenase was a precursor of the aerobic L-lactate–cytochrome *c* reductase. The presence of D-lactate cytochrome *c* reductase in aerobic yeast (Nygaard, 1960) lends support to this hypothesis and suggests that D-lactate cytochrome *c* reductase is an intermediate in the transformation. The extreme salt-sensitivity of D-lactate cytochrome *c* reductase recalls the extreme salt-sensitivity of L-lactate cytochrome *c* reductase as reported by Boeri, Cutolo, Luzzati and Tosi (1955) and by Boeri and Tosi (1956). The fact that L-lactate cytochrome *c* reductase isolated from our yeast is insensitive to salt suggests that more than one form of this enzyme can be isolated from aerobic yeast. One of the forms could be an intermediate in the development of the other.

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STUDIES ON BAKERS' YEAST LACTATE DEHYDROGENASE

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Yakushiji and Mori (1937) reported the extraction and partial purification of a '*b*-type cytochrome' from bakers' yeast. Using the preparation, Okunuki and Yakushiji (1940) proposed a scheme for the terminal electron-transferring system as follows:

$$\begin{tikzcd}
\text{Substrate} \arrow[rr] & & \text{Coenzyme} \arrow[rr] & & \text{`Cytochrome } b\text{'} \arrow[rr] & & \text{Cytochrome } c \arrow[rr] & & \text{Oxygen} \\
& \text{Dehydrogenase} \arrow[u] & & \text{Diaphorase} \arrow[u] & & & & \text{Cytochrome oxidase system} \arrow[u] &
\end{tikzcd}$$

Borei (1945) reported that 'cytochrome *b*' was not extracted according to the method of Yakushiji and Mori. On re-examination, the method, with slight modifications, was found suitable for the extraction of cytochrome *c* and cytochrome b_2, but not for the extraction of a typical cytochrome *b*. After the extraction of both cytochromes, the cytochrome *b* still remained in the cellular particles. The description by Yakushiji and Mori is therefore regarded by us as the first detection and partial purification of cytochrome b_2. Both cytochromes *c* and b_2 extracted from bakers' yeast have been crystallized (Hagihara, Horio, Yamashita, Nozaki and Okunuki, 1956; Yamashita, Higashi, Yamanaka, Nozaki, Mizushima, Matsubara, Horio and Okunuki, 1957).

Appleby and Morton (1954, 1959a, b) succeeded in crystallizing lactate dehydrogenase (Y–LDH) from bakers' yeast. Their crystalline Y–LDH (molecular weight, 80,000) contained riboflavin phosphate, deoxyribopolynucleotide (Morton, 1955, 1958; Appleby, 1957) and haem iron, but no non-haem iron. Moreover, the enzyme was capable of reducing cytochrome *c* and methylene blue at the expense of lactate in the absence of any coenzyme and showed absorption spectra just like a *b*-type cytochrome. In its lactate-reduced form the absorption peaks of the preparation were at 556 mμ (α-band), 527 mμ (β-band) and 423 mμ (γ-band), and in its oxidized form at 413 mμ (γ-band). This enzyme was called cytochrome b_2 by Bach, Dixon and Zerfas (1946) who partially purified the cytochrome simultaneously with increasing the specific enzymic activity. On the other hand, the cytochrome b_2 crystallized by us (Yamashita *et al.*, 1957) contained one haem iron atom in each 22,000 g protein, but no flavin nor non-haem iron. Moreover, it showed no enzymic activities in all tests tried in the presence and absence of flavin (flavinadenine dinucleotide, FAD; riboflavin phosphate, FMN; and riboflavin, RF). Hereafter in this paper, in order to avoid confusion, the enzymic and non-enzymic cytochromes will be called Y–LDH and cytochrome b_2, respectively.

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In addition to the method of Yamashita *et al.* (1957), by which cytochrome b_2 was directly extracted at pH 8·0 from the cells of bakers' yeast previously disrupted by ethyl acetate, it was found that cytochrome b_2 could be split from the Y–LDH purified according to the method of Bach *et al.* (1946) by treating it at high pH.

The present paper deals with some studies on relationships between the lactate dehydrogenase moiety and the cytochrome b_2 moiety, both of which seemed to be the main moieties of the whole enzyme, Y–LDH.

ENZYMIC ACTIVITIES OF YEAST LACTATE DEHYDROGENASE (Y–LDH)

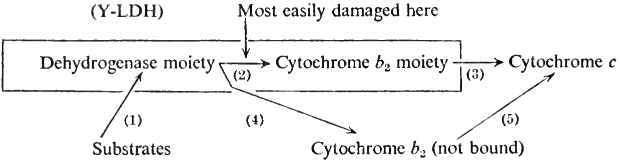
The preparation of Y–LDH purified mainly according to the method of Bach *et al.* (1946) has been found capable of oxidizing various substances other than lactate: α-glycerophosphate (Lehmann, 1938), glycerate (Dickens and Williamson, 1956), malate and reduced triphosphopyridine nucleotide, TPNH (Yamanaka, Horio and Okunuki, 1958), and α-hydroxybutyrate (Yamashita, unpublished). If these substrates are added to the enzyme preparation purified to a specific activity of about 6,000 units (as Q_{MB} at 30°C) Y–LDH immediately exhibits its reduced absorption spectrum to a variable extent, dependent on the substrate. With equal concentrations of the substrates, the extent of reduction is highest with lactate, somewhat lower with α-hydroxybutyrate, and very low with the others. The reduction by malate gradually increases as its concentration rises to the maximum examined (0·5 M). On the other hand, Y–LDH is known to be able to deliver electrons from the substrates to cytochrome *c*, methylene blue, ferricyanide, phenazine methosulphate, etc. The rates of reduction of these electron acceptors by the substrates vary in proportion to the extent to which Y–LDH itself is reduced by these substrates.

Relationship between Y–LDH and Cytochrome b$_2$

During the purification of Y–LDH, the sample is always contaminated with the cytochrome b_2 not bound to Y–LDH. If lactate is aerobically added in excess to such an enzyme preparation, all Y–LDH is immediately reduced, but the free cytochrome b_2 is only slightly reduced because of its high autoxidizability (Yamashita, Horio and Okunuki, 1958). Most of the free cytochrome b_2 can be removed from the enzyme preparation by taking advantage of different affinities of calcium phosphate gel for the two proteins.

Reductions of Cytochrome c *and Cytochrome* b$_2$ *by Lactate, α-Hydroxybutyrate and Malate in the Presence of Y–LDH*

Under anaerobic conditions at pH 6·4, cytochrome *c* is reduced by Y–LDH 42 times as fast as cytochrome b_2. When cytochrome *c* or cytochrome b_2 is used as a final electron acceptor of Y–LDH, reduction of cytochrome *c* by lactate, α-hydroxybutyrate, and malate shows optimal pH at pH 6·5, 5·5 and 8·0, respectively, while reduction of cytochrome b_2 shows optimal pH at about 5, 5, and 8–9, respectively. With lactate and α-hydroxybutyrate, activity–pH curves for the cytochrome *c* reduction reactions are fairly sharp and roughly symmetrical on both sides of each optimal pH. However, the activity–pH curves for the reduction of cytochrome b_2 by lactate and α-hydroxybutyrate are asymmetrical, showing appreciable activity even at pH 4·0 on the acid side of the pH optimum, but dropping steeply on the alkaline side. In contrast, the activity–pH curve for the reduction of cytochrome b_2 by malate is nearly constant between pH 8–9, and falls steeply at pH lower than 8. The remarkable differences between the activity–pH curves for the reduction of cytochrome *c* and cytochrome b_2 by the various substrates might be explained by the following pathway:



where the box expresses the whole enzyme, Y–LDH, consisting of two parts, the dehydrogenase and the cytochrome b_2 moieties. Reaction (1) expresses a dehydrogenase activity, and reaction (2) an electron transport in the Y–LDH complex. If the externally added cytochrome *c* is reduced by Y–LDH as fast as is the cytochrome b_2 moiety, then reaction (2) proceeds 42 times faster than reaction (4). In this scheme of electron transport, the intimate junction between the dehydrogenase and cytochrome b_2 moieties may be broken at the lower and higher pH ranges, and reaction (4) may take the place of reaction (2).

Modification of the Enzymic Activity of Y–LDH

When Y–LDH is allowed to stand at pH 7·0 and 4–5°C in the absence of the substrates, its enzymic activity gradually decreases. During storage, the ability to reduce methylene blue, ferricyanide and cytochrome *c* decreases much faster than does the ability to reduce phenazine methosulphate. Figure 1 shows a notable difference in the enzymic activities between the original enzyme and the enzyme stored for 72 hr (Horio, Yamashita and Okunuki, 1959). Such partial modification of Y–LDH can be demonstrated within a much shorter time if the storage is carried out at pH 5·0, at which pH it has been suggested (*vide supra*) that the electron-transferring pathway in Y–LDH might be most easily damaged between the dehydrogenase and cytochrome b_2 moiety. If lactate is aerobically added to the modified Y–LDH, the modified enzyme shows the reduced absorption spectrum of cytochrome b_2 only slightly or not at all, even at concentrations at which the original and modified enzymes show the same enzymic activity by the phenazine methosulphate method, and at which the dithionite-reduced absorption spectra of both samples are easily measurable. The modified enzyme has not yet been purified to a state free of cytochrome b_2.

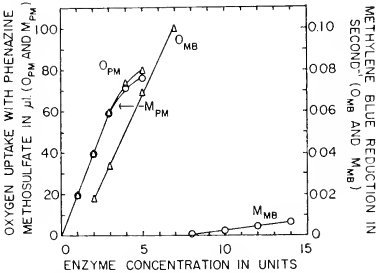


FIG. 1. Partial modification of bakers' yeast lactate dehydrogenase. The enzymic activities of the original and treated Y–LDH were assayed by two different methods: phenazine methosulphate method (curves, O_{PM} and M_{PM}), and methylene blue method (curves, O_{MB} and M_{MB}). Reduction of methylene blue by lactate was carried out in Thunberg tubes having an oxygen-free nitrogen gas phase at 35°C. The enzymic activities were compared between freshly-prepared Y–LDH (curves, O_{PM} and O_{MB}) and a sample of the Y–LDH stored in the absence of lactate at pH 7·0, and at 4–5°C, for 72 hr. One unit of Y–LDH (original and treated) was defined as the quantity of the enzyme capable of consuming 20 μl. of oxygen in the presence of phenazine methosulphate and lactate in the first 5 min at 38°C.

Protection by Substrates against the Inactivation of Y–LDH by Heat

Bach *et al.* (1946) found that lactate remarkably protects Y–LDH against heat-denaturation, and they adopt this property to their Y–LDH purification procedure. The other substrates can protect against denaturation, although they are inferior to lactate in their protective ability. The protective abilities of the various substrates vary in the same manner as do the rates at which the substrates are oxidized by Y–LDH: lactate, very strong; α-hydroxybutyrate, a little weaker than lactate; malate, very weak. Throughout the time course of the denaturation of Y–LDH by heat or by lowering the pH in the presence of either lactate of α-hydroxybutyrate, the reactivity of the enzyme toward the two substrates maintains a constant ratio. This is the case even if the activity is assayed with the use of phenazine methosulphate, ferricyanide, methylene blue, or cytochrome *c* (Yamashita, unpublished). This fact indicates that lactates and α-hydroxybutyrate are dehydrogenated not only by the same enzyme (Y–LDH), but also by the same dehydrogenase moiety (reaction (1)). Since Y–LDH shows much lower affinities for the other substrates as compared with lactate and α-hydroxybutyrate, it has been found difficult to apply this method to the dehydrogenation of the other substrates.

DISCUSSION

Boeri, Cutolo, Luzzati and Tosi (1955) and Boeri and Tosi (1956) have found that their best preparation of Y–LDH contains one FMN residue, one haem group and eight iron atoms not bound to haem for each 230,000 g protein, and they demonstrated the absence of a diphosphopyridine nucleotide-dependence for the dehydrogenase activity. Therefore they proposed the following scheme for electron transport in Y–LDH:

$$\text{Lactate} \longrightarrow \underbrace{\boxed{\text{FMN} \longrightarrow \text{non-haem irons} \longrightarrow \text{haem iron}}}_{\text{(Y–LDH)}} \longrightarrow \text{cytochrome } c$$

The extensive characterization of the crystalline Y–LDH by Appleby and Morton (see Morton, 1955, 1958; Appleby and Morton, 1954, 1959a, b) may require some modifications of the scheme as follows:

$$\text{Lactate} \longrightarrow \underbrace{\boxed{\text{riboflavin phosphate} \longrightarrow \text{haem iron}}}_{\text{(Y–LDH)}} \longrightarrow \text{cytochrome } c$$

Moreover, the successful crystallization of Y–LDH may support the concept that both dehydrogenation of an organic compound and the transport of the resulting electrons to a cytochrome can be carried out by one enzyme.

The linkage between the dehydrogenation of succinate and the reduction of cytochrome *b* may fit this concept. Singer and co-workers (Singer, Kearney and Bernath, 1956; Singer, Massey and Kearney, 1957) have succeeded in preparing mammalian succinate dehydrogenase in a truly homogeneous, water-soluble state. Their dehydrogenase contains flavin dinucleotide and non-haem iron(s). The purest enzyme can oxidize succinate with the aid of phenazine methosulphate, but not with methylene blue and ferricyanide, while in the crude state the enzyme can reduce all of them. On the other hand, cytochrome *b* has been purified in an apparently water-soluble state with the aid of detergents and proteinase digestion (Sekuzu and Okunuki, 1956). Attempts to link cytochrome *b* with the solubilized succinate dehydrogenase have not yet succeeded. Chance (1954) has assumed that the electron transport between the succinate dehydrogenase and cytochrome *b* may be mediated by another flavoprotein, but all attempts at separation of such a flavoprotein have been unsuccessful. Keilin and King (1958) have demonstrated that the water-soluble succinate dehydrogenase purified by the method of Wang, Tsou and Wang (1956) can transfer electrons liberated from succinate to the cytochrome system contained in a mitochondrial particle preparation which has no succinate dehydrogenase. The similarity in the modification of the enzymic activities between Y–LDH and succinate dehydrogenase might indicate similarities in their electron-transferring systems (compare, however, Morton, 1955).

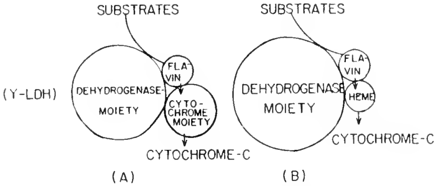


FIG. 2. Models of bakers' yeast lactate dehydrogenase. Schematic representations of the relationship between bakers' yeast lactate dehydrogenase activity and the reduction of the haem of bakers' yeast lactate dehydrogenase.

At present, the two models of Y–LDH may be considered to be as shown in Fig. 2, where (*A*) indicates that Y–LDH is a protein complex which is composed of a cytochrome plus a dehydrogenase containing a bound flavin. In this model the dehydrogenase moiety may be equivalent to the modified Y–LDH or to solubilized succinate dehydrogenase and the cytochrome moiety may be equivalent to crystallized cytochrome b_2 or to highly purified cytochrome *b*. Other concepts of an enzymic protein complex may be deduced from studies of phosphorylase *a* and *b* (Green and Cori, 1943) and L-amino acid oxidase (Blanchard, Green, Nocito and Ratner, 1944, 1945, 1946). At present, however, there is no evidence to deny the scheme (*B*), where the dehydrogenase protein itself holds both the *b*-type haem and a flavin, except the observation that: Y–LDH and cytochrome b_2 show identical α-, β-, γ- and δ-absorption maxima (in wavelength and in ratio). This is in contrast to the behaviour of cytochrome *c*, for native cytochrome *c* and cytochrome *c* digested by proteolytic enzymes are spectrophotometrically different, though the native cytochrome *c* is not spectrophotometrically distinguished from the cytochrome *c* modified only in its secondary structure (Nozaki, Yamanaka, Horio and Okunuki, 1957; Nozaki, Mizushima, Horio and Okunuki, 1958; Mizushima, Nozaki, Horio and Okunuki, 1958; Yamanaka, Mizushima, Nozaki, Horio and Okunuki, 1958). Insofar as cytochrome b_2 and cytochrome *c* are comparable, this behaviour may indicate that the bound cytochrome b_2 in the Y–LDH complex and purified cytochrome b_2 are identical, except perhaps with regard to secondary structure.

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DISCUSSION

The Problem of Cytochrome b_2

DICKENS: We now come to the Discussion of these very interesting papers and it has been suggested that, as Chairman, I should summarize briefly the main points which have arisen so far. I am bound to say that I find this a virtually impossible task, but I shall try to bring out a few points, which the authors of the papers presented are free to put right if they do not agree. In my view, there are first of all the three groups headed by Morton (Australia), Boeri (Italy) and Nygaard (Norway). The first two of these have no really essential difference in regarding the yeast enzyme as a flavohaemoprotein and, whether they prefer to call it 'cytochrome b_2' or 'flavocytochrome b_2', these authors are satisfied that they are dealing with a single unit containing protohaem, flavin and a single enzyme protein, though these compounds can be caused to dissociate under some treatments. There are, however, points of difference between these groups of authors, particularly in regard to the behaviour of the enzyme with different terminal acceptors, and also in regard to substrate specificity.

Morton and co-workers find that their well-crystallized cytochrome b_2 is almost free from non-haem iron and is only slightly autoxidizable while Boeri and Rippa find that their uncrystallized but highly active material is autoxidizable especially at higher oxygen concentrations, but that this autoxidizability is inhibited by excess lactate, probably by binding of active metal (Fe) ions by substrate. Morton and co-workers find that dissociation of part of the flavin increases autoxidizability of the crystalline material, with a decline in activity towards ferricyanide. The best substrate is L-lactate, though oxidation of some other α-hydroxy acids (but not glycerate) occurs, as reported by Morton and co-workers.

Nygaard is evidently dealing with a very different material, or rather three different non-crystalline materials, prepared from Norwegian yeast. By purification including DEAE-cellulose adsorption, he has obtained these fractions with different activities and different heat-stabilities, all able to oxidize lactate, though with differences in reactivity towards different hydrogen acceptors and in optical specificities. Whereas Morton's preparation contained some deoxyribonucleotide, Nygaard's had polyribonucleotides, retained even after dialysis. How much these preparations represent breakdown during extraction is not yet clear.

The Japanese workers also have made studies of this enzyme. Ogura's kinetic studies establish that the flavin group is involved in the enzymic reaction, supporting the original work of Morton's group. However, Hasegawa and Ogura do not seem to agree entirely with Morton and co-workers on the reaction with ferricyanide. The Japanese work at Osaka demonstrates the alkaline extraction from Japanese yeast of a crystalline haemoprotein having an absorption spectrum resembling the other workers' cytochrome b_2, but devoid of lactate dehydrogenase activity, and having no flavin or non-haem iron. It was also formed by alkaline treatment of a 'yeast lactate dehydrogenase' of the flavohaemoprotein type. Horio and co-workers call this haemoprotein 'cytochrome b_2' and suggest that in the intact system a dehydrogenase, containing a bound flavin, is linked to their cytochrome b_2, somewhat similarly to the composition of succinate oxidase. The substrate specificity of the partially-purified complex is wider than that observed by Morton and co-workers with their material, and includes glycerate, malate and TPNH.

While this enzyme system is of a novel and most interesting type, there is one question that I would like to put to the various contributors: what is the metabolic function of L-lactic dehydrogenase in the intact yeast cell? Lactate is not generally considered to be an important metabolite in yeast, although it is known that *added* lactate can be freely utilized (Meyerhof, *Biochem. Z.* **162,** 43, 1925). There is obviously a problem here.

My second point concerns substrate specificity. This varies in the different preparations studied, and the reason for this interests me very much. With the original preparation of Bach, Dixon and Zerfas, we found (Dickens and Williamson, *Nature, Lond.* **178,** 1118, 1956) that at pH 6·7 glycerate was oxidized to hydroxypyruvate at half the rate of lactate; only the L-isomer was attacked, and a purified yeast lactate dehydrogenase kindly supplied by Boeri gave a similar result. This raises the possibility that an α-hydroxyacid other than lactate might be the natural substrate.

Finally I would like to add a correction to a proposed mechanism for enzymic dehydrogenation of lactate and glycerate to pyruvate and hydroxypyruvate (Dickens and Williamson, *Biochem. J.* **68,** 74, 1958).

Because our crystalline lithium salts of these two acids occur as monohydrates, and infra-red spectroscopy by Bellamy and Williams (*Biochem. J.* **68**, 81, 1958) failed to detect the presence in either solid salt of a keto group or of a molecule of water of crystallization, we tentatively suggested a hydration of the keto- to a diol-form as part of the enzymic reaction. While this is still not impossible, recent studies kindly carried out by D. A. Long (of University College, Swansea) on the Raman spectra in aqueous solution do not support the above findings based on the solid substances; in fact they indicate predominantly a keto-form in aqueous solutions of lithium or sodium pyruvate. So far, fluorescence difficulties have prevented similar observations on hydroxypyruvate solutions.

Properties of Intact and Modified Cytochrome b_2

Nature of Bakers' Yeast Lactate Dehydrogenase

By T. HORIO (Osaka)

HORIO: I would like to consider again the true physiological function of bakers' yeast lactate dehydrogenase. Of course, the enzyme extracted and purified from bakers' yeast can dehydrogenate lactate and other substrates: namely, α-hydroxybutyric acid, and exceptionally TPNH. When these substrates are added to the enzyme solution, the cytochrome b_2-moiety bound on the enzyme is reduced. Of the various substrates tested, lactate is most rapidly attacked by this enzyme, so most workers use lactate as its substrate. However, using cytochrome *c* in its reduced form as an electron donor, the back reaction from pyruvate to lactate has never been demonstrated to take place physiologically, as reported by Boeri and co-workers. Furthermore, any metabolic pathway to produce lactate has never been found in bakers' yeast, as far as I know. From this point of view, I am just wondering the reason why bakers' yeast cells have such a lot of lactate dehydrogenase.

When purified, bakers' yeast lactate dehydrogenase is quite water-soluble. However, our procedure for its extraction from bakers' yeast cells is very complex and different from that of other workers. In our experiments, pressed bakers' yeast is disrupted by a minimum amount of ethyl acetate, then washed with a great deal of water. The washed cells are extracted with 20%-saturated ammonium sulphate at a neutral pH. Most of cytochrome *c* can be extracted in the salt solution by this extraction, but almost none of the enzyme by the procedures up to this step. This debris is suspended again in water, then the enzyme comes out of the debris to a great extent.

When the activity of bakers' yeast lactate dehydrogenase purified mainly according to the method of Bach, Dixon and Zerfas (*Biochem. J.* **40**, 229, 1946) is assayed by the phenazine methosulphate-method of Singer and co-workers, and by methylene blue-, ferricyanide- and cytochrome *c*-methods with the use of lactate as the substrate, the ratio of the phenazine methosulphate activity to the methylene blue, etc., activities varies for different enzyme preparations. As a rule, we might say that the more native the enzyme is, the lower is the ratio of phenazine methosulphate activity to methylene blue activity (see Fig. 1, Horio, Yamashita, Yamanaka, Nozaki and Okunuki: this volume, p. 555).

As we have already pointed out, the modification of yeast lactate dehydrogenase which occurs on various treatments suggests to us the scheme of electron transport which may be broken at different sites (see Horio *et al.*, this volume, p. 554). The intimate junction between the 'dehydrogenase' and 'cytochrome b_2' moieties may be broken at the lower and high pH ranges, and the reaction (4) may take the place of reaction (2).

It may be thought that the differences of the results presented by Ogura, Nygaard, Boeri, Morton and ourselves are dependent upon these changeable properties of the enzyme itself, as well as the original source of bakers' yeast cells as suggested by Nygaard. To my regret, I have not yet succeeded in crystallizing this enzyme although I have used four kinds of yeast cells made in Japan, and although our extraction method works well with all these yeasts. Therefore, we have been compelled to use a rather crude enzyme preparation for this experiment.

Figure 1 shows some mutual relationships of cytochrome b_2, flavin, and phenazine methosulphate and methylene blue activities during the course of the modification of the enzyme at a lower pH (Yamashita, Horio and Okunuki, unpublished). The curve 'cytochrome b_2' was made by dithionite-reduction of the α-absorption maximum of cytochrome b_2. The curve 'flavin' was measured by fluorescence with the use of supernatant of an ammonium sulphate-saturated sample. Because of the use of a crude enzyme preparation, I think that the amount of liberated flavin does not correctly show the inter-relationship with the enzyme modification. However, it may be assumed that the liberation of flavin has a certain important role in the partial modification of bakers' yeast lactate dehydrogenase, as indicated by Appleby and Morton (*Nature, Lond.* **173**, 749, 1954; *Biochem. J.* **73**, 539, 1959).

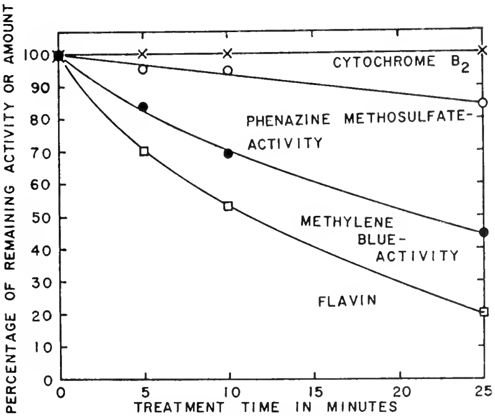


FIG. 1. Modification of bakers' yeast lactate dehydrogenase. The figure compares the percentage of remaining activity with phenazine methosulphate and with methylene blue after treatment of the enzyme at low pH, with the amount of remaining dithionite-reducible haemoprotein (expressed as 'cytochrome b_2') and the amount of liberated flavin (measured as fluorescence).

Compared with the enzyme, the 'cytochrome b_2' (split from the enzyme) is very stable, and the cytochrome so formed can be easily crystallized. The experimental conditions for direct extraction of cytochrome b_2 from bakers' yeast cells, involving as it does extraction at a higher pH (pH 8) and other results, clearly indicate that 'cytochrome b_2' does *not* exist independently apart from the enzyme, at least in its physiologically native state. In order to investigate the mode of existence of cytochrome b_2 in the enzyme, the absorption spectra of cytochrome b_2 and the enzyme in the visible wavelength were compared, and it was found that there is no notable difference above experimental error. If cytochrome *c* is used as a model case, variously different kinds of cytochrome *c*'s can be easily prepared: a native one having its coiling structure, a modified one damaged in the coiling structure, and a proteolysed one. The former two show the same absorption spectra which are remarkably different from the latter. This means that the absorption spectrum of cytochrome *c* is not seriously changed by damaging the coiling structure, but is remarkably altered by shortening its amino acid composition (see Horio *et al.*, this volume, p. 557, for references). Analogously considered, the following two kinds of existence of cytochrome b_2 might be assumed. In (1), 'cytochrome b_2' having no flavin and no enzymic activity, exists in a state in which it has its own protein configuration separate from the dehydrogenase moiety of bakers' yeast lactate dehydrogenase, and forms a protein complex with the dehydrogenase moiety to be physiologically active. In (2) bakers' yeast lactate dehydrogenase itself holds a *b*-type haem and a flavin. Then, if this enzyme is treated at a higher pH or some other drastic conditions, the enzyme protein is modified to liberate the flavin but not the *b*-type haem. The two models of bakers' yeast lactate dehydrogenase may be considered to be as shown in Fig. 2 of our paper (Horio *et al.*, this publication, p. 557), which we have already discussed.

Here I would like to recall again the case of succinate dehydrogenase. As is well known, the extraction procedure of the enzyme is rather complex. The treatment of the starting source with organic solvent such as n-butanol (Morton, *Nature*, *Lond.* **166,** 1092, 1950) causes a serious decrease of total activity of the enzyme. However, the enzyme can be easily extracted after this treatment, but not before the treatment. Slater discussed with Singer at the International Symposium on Enzyme Chemistry held in Japan (Proceedings of the International Symposium on Enzyme Chemistry, 1957) this point. The activity of succinate dehydrogenase to phenazine methosulphate, ferricyanide, methylene blue, etc., changes during purification steps. Slater questioned the inter-relationships between the activity-changes and the iron bound on the enzyme. Even in a most crude state, the solubilized succinate dehydrogenase cannot join with the purified cytochrome *b*. These things recall the same question as for bakers' yeast lactate dehydrogenase: what is the native succinate dehydrogenase and what is cytochrome *b*? From this point of view, the activity changes of succinate dehydrogenase is quite similar to the activity changes of bakers' yeast lactate dehydrogenase. At the same time, there might be a great doubt about whether or not so-called 'cytochrome *b*' does exist in a free state independent from the 'physiologically native' succinate dehydrogenase. This same question might be raised in relation to diaphorase and cytochrome c_1. In fact, during the purification of cytochrome c_1 with the use of cholate, an intimate connection is observed between both components, and initially DPNH can reduce cytochrome c_1. However, this enzymic reaction is broken as the purification procedure goes on, and purified diaphorase and cytochrome c_1 do not react with each other.

Very recently, Iwatsubo and Kubo (Medical School, University of Osaka, Osaka; personal communication) succeeded in demonstrating an interesting flavin-level reaction, using D-amino acid oxidase and xanthine oxidase which have been completely purified by them. In an anaerobic condition, in the presence of both enzymes, the electrons are transferred from D-amino acid to cytochrome *c*. This reaction may be demonstrated regardless of the use of apo- or holo-D-amino acid oxidase. In fact, under an experimental condition in which xanthine oxidase is perfectly stable and in which flavin is not liberated from the enzyme, the apo-enzyme of D-amino acid oxidase can consume oxygen in the presence of its substrate and xanthine oxidase. Their experiments may illuminate the flavin–cytochrome electron transferring system in respiration.

BOERI: With reference to the observations of Horio and co-workers, and of Nygaard, we also have found that phenazine methosulphate is a good acceptor with flavocytochrome b_2, and it may be used in an activity test. The test may be carried out manometrically, by following the oxygen consumption for reoxidation of phenazine methosulphate, as used for succinate dehydrogenase by Singer and Kearney. The addition of cyanide is unnecessary, as no hydrogen peroxide is formed.

The test may also be performed spectrophotometrically by measuring the change in extinction at 387 mμ. We use a molar extinction of $2{\cdot}27 \times 10^5$ for the oxidized dye, and of $1{\cdot}62 \times 10^3$ for the leuco compound. It is not necessary to use anaerobic cuvettes but it *is* necessary to use air-free solutions.

Nomenclature of Cytochrome b_2 *and Derived Proteins*

MORTON: The various terms used in the several papers related to yeast lactate dehydrogenase and cytochrome b_2 calls for some comment, lest the confusion become too great.

Historically, the name 'cytochrome *b*$_2$' was given by Dixon and co-workers (Bach, Dixon and Keilin, *Nature, Lond.* **149,** 21, 1942; Bach, Dixon and Zerfas, *Biochem. J.* **40,** 229, 1946) to a haemoprotein seen in yeast extracts, which was reduced in the presence of lactate and oxidized by cytochrome *c* and by oxygen. If we retain the prefix 'cytochrome'—given by Keilin to naturally-occurring intracellular haemoprotein pigments capable of reversible oxidation and reduction—then the name 'cytochrome *b*$_2$' should be used for the intact, homogeneous protein containing haem and flavin groups which was first isolated and described by Appleby and Morton (*Nature, Lond.* **173,** 749, 1954). This crystalline protein has L(+)-lactate dehydrogenase activity. The evidence in favour of this view has been set out by Appleby and Morton (1954, *loc. cit.*; *Biochem. J.* **71,** 492, 1959; and *Biochem. J.* **73,** 539, 1959) and by Morton (*Society of Biological Chemists, India, Silver Jubilee Souvenir*, p. 177, 1955; *Rev. pure appl. Chem.* **8,** 161, 1958).

Although we hold the view that the polydeoxyribonucleotide (Morton, 1958, *loc. cit.*; Appleby and Morton, *Biochem. J.* **73,** 539, 1959; *Biochem. J.* **75,** 258, 1960), is an integral component of the naturally-occurring material, as yet there is no evidence that this polynucleotide has any direct function in relation to the enzymic activity. Hence the *minimum* requirement for lactate dehydrogenase activity is the flavohaemoprotein.

Boeri refers to this flavohaemoprotein having enzymic activity as 'flavocytochrome *b*$_2$', but there seems no good reason for the addition of 'flavo' other than to distinguish this material from derived proteins devoid of the flavin group.

Okunuki and co-workers refer to 'cytochrome *b*$_2$' as a haemoprotein, devoid of L(+)-lactate dehydrogenase activity, which they isolated from yeast cells (Yamashita, Higashi, Yamanaka, Nozaki, Mizushima, Matsubara, Horio and Okunuki, *Nature, Lond.* **179,** 959, 1957). The behaviour of this material as reported by Okunuki and co-workers (see Horio, this volume, p. 552 and p. 560), and our own observations (Morton, 1958: *loc. cit.*, Morton, Armstrong and Appleby, this volume, p. 501; Armstrong, Coates and Morton, this volume, p. 571) indicate that this haemoprotein is modified from intact cytochrome *b*$_2$. The relationship of this material to intact cytochrome *b*$_2$ is somewhat analogous to the relationship of the peptic (or tryptic) 'core' of heart-muscle cytochrome *c* to cytochrome *c*. I think that no one would refer to the 'core' as 'cytochrome *c*'. I therefore consider that the enzymically-inactive haemoprotein derived from cytochrome *b*$_2$ should not be called 'cytochrome *b*$_2$' but some other name, possibly 'haemoprotein 557 from cytochrome *b*$_2$', which would have the value of indicating the position of the α-absorption band and the relationship to cytochrome *b*$_2$

As suggested by the observations of Slonimski and colleagues (Slonimski and Tysarowski, *C. R. Acad. Sci., Paris* **246,** 1111, 1958; Labeyri, Slonimski and Naslin, *Biochim. biophys. Acta* **34,** 262, 1959) and of Nygaard (*Biochim. biophys. Acta* **35,** 212, 1959 and this volume, p. 544), it is likely that there is more than one 'lactate dehydrogenase of bakers' yeast', particularly if the yeast is grown under partially-anaerobic conditions. The term 'lactate dehydrogenase' refers to the protein which specifically activates lactate for dehydrogenation. Cytochrome *b*$_2$ is one such dehydrogenase (certainly the principal L(+)-lactate dehydrogenase of aerobic bakers' yeast) and the term 'cytochrome *b*$_2$' is therefore preferable to the less-specific 'lactate dehydrogenase of bakers' yeast' when the flavohaemoprotein is implied.

The Substrate Specificity of Cytochrome b$_2$

BOERI: With reference to our non-crystalline preparations of cytochrome *b*$_2$, and the crystalline preparation obtained by Appleby and Morton, the following points should be noted.

(a) As found by Dickens and collaborators, our preparations dehydrogenate glycerate whereas the preparations of Morton and co-workers (Morton, Armstrong and Appleby, this volume, p. 501) do not.

(b) Our preparations are inert to both isomers of malate, whereas the crystalline preparations of Morton and colleagues are inhibited slightly by L-malate and strongly by D-malate (Morton *et al.*, this volume, p. 501).

It should be noted that both our preparation, and that of the Australian workers, fail to dehydrogenate reduced triphosphopyridine nucleotide (TPNH), whereas this is dehydrogenated by the preparation of Okunuki's school (Horio, Yamashita, Yamanaka, Nozaki and Okunuki, this volume, p. 552).

With reference to observations of Nygaard, in my experience the cytochrome b_2 acts specifically on L(+)-lactate. Another enzyme of the yeast, present in anaerobic yeast, was shown by Slonimsky and his collaborators to act on D(−)-lactic acid. It is not a flavo-cytochrome, but a flavoprotein. Work is going on in our laboratory on this enzyme which we prefer to define, with a terminology different from that of Slonimsky, as a D-hydroxyacid dehydrogenase.

ARMSTRONG: The substrate specificity of crystalline cytochrome b_2 was shown by Appleby and Morton (*Biochem. J.* **73**, 539, 1959) to include glycolate and α-hydroxybutyrate. The further studies of Armstrong and Morton (see Table 5, this volume, p. 512) show that a number of α-hydroxymonocarboxylic acids are substrates. Additional hydroxycarboxylic and certain other groups probably interfere with attachment if sufficiently close to the α-hydroxyl group. In D-malate, the hydroxyl group has the same configuration as in L-lactate if viewed from the β-carboxyl end. Thus it is not surprising that D-malate is a much more powerful inhibitor of the reaction with L-lactate than is L-malate. One might expect that certain esters of malate might be substrates of cytochrome b_2. It would appear from the pH optima reported by Horio, Yamanaka, Yamashita, Nozaki and Okunuki (this volume, p. 552) that malate and TPNH are dehydrogenated by enzymes other than cytochrome b_2 in the preparation used by these workers.

On the Cytochrome b *Components in the Respiratory Chain in Yeast*

CHANCE: The following relates to the possible function of cytochrome b_2 in the respiratory chain in yeast. In 1954 we were actively considering the possibility that the *b* component of yeast cells could be identified with components ranging from cytochrome b_2

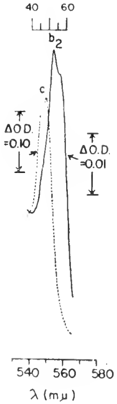


FIG. 1. Spectrum of reduced cytochrome b_2 compared with that of cytochrome *c* at liquid air temperatures (RE-129a).

to peroxidase (Chance, 1954: In *Mechanisms of Enzyme Action*: Johns Hopkins Press, Baltimore, p. 399). We have, however, many new data to offer on this point, particularly low temperature data on cytochrome b_2 (Chance, Klingenberg and Boeri, *Fed. Proc.* **15**, 231, 1956). It has been found that the low temperature spectrum of reduced cytochrome b_2 is easy to distinguish from that of cytochrome *b* of the bakers' yeast cells. The former is illustrated by Fig. 1 which shows that the α_1- and α_2-bands of cytochrome b_2 lie at 558 and 553·4 mμ. In the low temperature spectrum of yeast cells (Chance, this volume, p. 233) the cytochrome *b* of the respiratory chain has an α peak at a longer wavelength, about 560 mμ, and no α_2 peak. These two curves clearly distinguish the two cytochromes and show that cytochrome b_2 is not involved to a measurable extent in the respiratory chain of yeast cells.

The Kinetics of Reactions Catalysed by Cytochrome b$_2$

The Kinetics of Reduction of Cytochrome b_2

By B. Chance (Philadelphia)

Chance: In spite of the great progress that has been made in the preparation of types of lactate dehydrogenase that exhibit various differences in their electron acceptor specificity, little experimental evidence is at hand to indicate the extent to which the haemoprotein portion of this enzyme participates in the electron transfer process. A single experiment is reported here in which the maximum speed with which lactate alone can reduce the haemoprotein component is recorded.

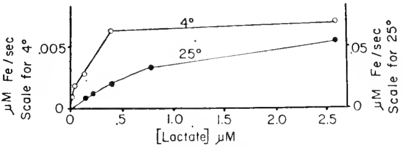


Fig. 1. Relationship between the concentration of lactate and the rate of appearance of the absorbance band of reduced cytochrome b_2 at 4°C and 25°C. Double-beam spectrophotometer (424–404 mμ); 0·15 M phosphate buffer (pH 8·5). At 4°C, the rate of absorbancy change of cytochrome b_2 proceeds at 0·16 μM Fe/sec; at 25°, the rate is 0·08 μM Fe/sec. Lactate concentrations appear on the scale of the abscissa (calculated as 0·5 DL-lactate concentration). Rates calculated for $\Delta\varepsilon$ 424 − 404 = 150 cm^{-1} × mM^{-1} (Expt. 540).

The results, illustrated by Fig. 1, show the relationship between the lactate concentration and the rate of increase of absorbancy measured at 424 mμ with reference to 404 mμ. The absorbancy changes are computed as μmoles of iron/l./sec with the approximate extinction coefficient (for this preparation, a value of approximately 150 cm^{-1} × mM^{-1} is used for 424 and 404 mμ). The important feature of the experiment is that the rate of reduction of the haemoprotein becomes independent of the lactate concentration at concentrations above 0·5 μM at the lower temperature, and above 2·5 μM lactate at the higher temperature. If the rate of reduction of the haemoprotein is divided by its concentration to get an effective value of turnover number, values of 0·04 and about 1 sec^{-1} are obtained at 4°C and 25°C respectively. Since it is clear that this value is so inferior to the value observed in ferricyanide reduction (about 220 sec^{-1} at 26°C) and the comparable value in cytochrome *c* reduction, we suggest that direct intramolecular electron transfer from flavin to haem is too slow to function in a pathway for the overall enzymic activity.

It may then be questioned whether there is intramolecular electron transfer at all, and suggested that the reduction of the haematin may be a purely intermolecular reaction, and this point requires further experimentation. In the presence of electron acceptors such as ferricyanide, the rate of reduction of the haematin is somewhat more rapid and it is feasible that the intermolecular electron transfer is facilitated under these conditions.

In summary, the flavohaemoprotein cytochrome b_2 shows only a slow rate of haematin reduction on adding lactate in the absence of electron acceptor. The system lacks the property of rapid intramolecular electron transfer characteristic of the intact respiratory chain, and more resembles the sluggish reduction of cytochrome *b* of the non-phosphorylating respiratory chain.

On Intramolecular Transfer in Cytochrome b_2

OGURA: Although Chance suggested by using rapid spectrophotometric method that the ferricyanide may be reduced through the protohaem of this enzyme, the same result was obtained also in our experiments by the method of overall reaction kinetics. The relationships between $1/v$ and $1/[s]$ which were obtained in the presence of low concentrations of ferricyanide were the same as shown in Fig. 4 (B) in the paper of Morton and co-workers (p. 517), namely, these relationships obtained seemed to be parallel to each other.

Although this result obtained by us does not seem to accord with the data of Morton and co-workers (this volume, p. 518) such discrepancy seems to be caused by the concentration of ferricyanide used. The lower concentrations of ferricyanide were used in our experiments, since the reaction at the initial stage was zero order in respect to the H-acceptor concentration in the presence of 0·5 mM ferricyanide.

MORTON: The discrepancy between the results obtained by Armstrong and myself, and those of Hasegawa and Ogura, can most probably be attributed to the difference in concentration range of ferricyanide, as suggested by Ogura. Our kinetic studies have to be extended, and we plan to examine the kinetics of the reaction of crystalline cytochrome b_2 at the lower concentrations of ferricyanide.

Although Fig. 1 of our paper (this volume, p. 502) shows ferricyanide reacting only with the riboflavin phosphate group, there is reason to believe that ferricyanide may react with either, or both, haem and riboflavin phosphate, according to the conditions of the experiment. This appears to be the situation also in the succinate dehydrogenase system.

We have been very much interested in the kinetic results obtained with the double-beam instrument since the original report by Chance, Klingenberg and Boeri, *Fed. Proc.* **15,** 231, 1956) of their studies with cytochrome b_2. Kinetic studies with this flavohaemoprotein are extremely difficult because of the lability of the material and the results are very much dependent on the conditions of the experiment. In the studies reported here (Morton, Armstrong and Appleby, this volume, p. 501), Armstrong and I used freshly-crystallized enzyme, kept under anaerobic conditions and in the presence of ethylenediamine tetra-acetate (EDTA). Crystallization is a useful tool in that it enables one to obtain high concentrations of enzyme relatively free of other materials. The material was non-fluorescent, indicating that both the flavin and the haem groups were intact on the protein.

Whenever such material is oxidized, it rapidly shows some fluorescence indicating some modification (denaturation) of the cytochrome b_2. For this reason we have found it very difficult to carry out reliable experiments with oxidized cytochrome b_2.

With reference now to the experiment reported here by Chance (p. 565), from our experience it would be essential (a) to exclude oxygen completely from the system; (b) carry out the experiment in the presence of 10^{-4} M EDTA (or similar metal-binding agent); and (c) use only native non-fluorescent cytochrome b_2. Not only does the presence of oxygen increase the rate of appearance of fluorescence, but also the rate of reaction of the cytochrome b_2 with oxygen is dependent on the degree of denaturation. As already noted (see Morton *et al.*, this volume, p. 501; Horio, Yamashita, Yamanaka, Nozaki and Okunuki; this volume, p. 552), the flavin-deficient haemoprotein is readily autoxidizable whereas the intact enzyme is only very slightly autoxidizable. Moreover, the rate of autoxidation of the intact enzyme is dependent on the concentration of lactate (Boeri and Rippa, this volume, p. 524; Morton *et al.*, this volume, p. 513). Clearly the amount of change at 424 mμ recorded by Chance (this volume, p. 565) would be dependent on the amount of oxygen present in the system.

Secondly, if the material used was not completely intact then the portion of intact native protein would react rather sluggishly with the haematin of the portion of denatured material. In this case, one would find only a slow rate of haematin reduction on adding lactate to the preparation. The difference in the behaviour of intact, non-fluorescent, crystalline cytochrome b_2, and denatured cytochrome b_2 indeed recall the differences in the reactions of cytochrome *b* in the intact mitochondrial system as compared with the reaction in the modified, non-phosphorylating fragments of sarcosomes.

Hence, at the present time, I feel that the conclusions of Chance (p. 565), that the reaction of the flavin with the haematin of cytochrome b_2 is intermolecular rather than intramolecular, must be treated with caution. The opposite conclusion reached in our studies (Morton *et al.*, this volume, p. 519) may possibly be attributed to the difference in the relative intactness of the preparations of cytochrome b_2 used, and to the conditions of the experiments.

CHANCE: The turnover number of the preparation was 220 sec^{-1} at 26°C.

The Contribution of the Prosthetic Groups to the Absorption Spectrum of Cytochrome b_2

MORTON: The extinction coefficients (expressed on the basis of millimolar concentration of protein-bound haem) are appreciably greater for the absorption bands of both reduced and oxidized cytochrome b_2 than for any other *B* group cytochrome as yet described (see Table 5, Morton, *Rev. pure appl. Chem.* **8**, 161, 1958). This is not merely due to the occurrence of narrow, high absorption bands. The same picture would emerge if areas under the bands were considered or extinctions were plotted against frequency rather than wavelength. In the reduced cytochrome b_2, the positions of the α-, β- and γ-bands (at room temperature) are very close to those of pyridine protohaemochrome.

As shown by Table 4 of our paper (Morton, Armstrong and Appleby, this volume, p. 509), nucleotide-free cytochrome b_2 has similar absorption bands in the visible portion of the spectrum, but removal of the polydeoxyribonucleotide causes a shift of the U.V. absorption band from 265 mμ to about 268 mμ, and a decline of ε_{mM} from about 199 at 265 mμ to about 135 at 268 mμ. In the flavin-free haemoprotein derivative, the U.V. maximum shifts to about 278 mμ and there is a further decline in ε_{mM}. Hence the flavin makes a significant and readily-detectable contribution to the absorption spectrum of cytochrome b_2 in the U.V. region. In the visible region, however, the effects of the flavin group on the absorption spectrum are not so apparent. The influence of the flavin group can be seen in the region of the δ-band and at about 450–470 mμ by comparison of reduced and oxidized cytochrome b_2 (see Figs. 2 and 3 of Morton *et al.*, this volume, p. 501; also Appleby and Morton, 1954, 1959, *loc. cit.*). In the fully oxidized material (Fig. 2) and in the reduced material at pH 10 (Fig. 3), however, much of the flavin would have dissociated from the enzyme. Hasegawa and Ogura (this volume, p. 534) have provided direct evidence of the contribution of riboflavin phosphate to the absorption spectrum since the difference spectrum (oxidized minus reduced) of their dye-treated enzyme is that of oxidized flavin.

The transient shift in the absorption bands of the lactate-reduced cytochrome b_2 when oxidized by air from approximately 557 and 528 mμ to 567 and 533 mμ respectively provides evidence of the formation of a 'lactate'—cytochrome b_2 complex, since the fully-reduced and fully oxidized compounds show no such unusual absorption bands, (see Appleby and Morton, 1959, *loc. cit.*, and Morton *et al.*, this volume, p. 510).

In order that such a complex may be formed and the absorption bands of the haemoprotein be affected, there must be a close juxtaposition of the haem and the flavin groups on the protein so that direct interaction may occur between these groups in the presence of 'lactate', thus accounting for the shift of the α- and β-bands of the fully-reduced cytochrome b_2. The considerable elevation of the extinction coefficients of the absorption bands of cytochrome b_2 as compared with other *B* group cytochromes may also indicate some type of interaction of the flavin group with the haem group. Another possible explanation, as suggested by Kamen, is that the enzyme exists as a polymer of the monomeric structure of weight 80,000 g/mole, and that the elevation of the absorption bands is due to the effects of polymerization.

DRABKIN: I would like to speak to Morton's question concerning possible evidence in favour of his most interesting model of cytochrome b_2 structure in the bonding of the protein with the protohaemin nucleus, namely his proposal that the isoalloxazine nucleus is sufficiently close to the haemin so as to influence it. What I say applies only to the spectrum, not to the role of the substance as an enzyme.

As Chance knows, I have been anxious to re-examine cytochrome b_2 spectroscopically. Morton's published spectrum of the reduced form was most provocative from the viewpoint of additional light it shed on my analytical procedure (this volume, p. 142). There were four main points of interest:

(1) There was the band at 260 mμ, certainly due to the riboflavin phosphate, which did not belong to my postulated main series. (2) There was nearly complete masking of the so-called protein peak at 280 mμ (my No. 8 band), although there was a very slight inflection in this region. (3) There was an anomalous band at about 475 mμ. (4) Above all, all the maxima, α, β, γ (or No. 6) and δ (or No. 7) were appreciably elevated in comparison with corresponding maxima in the more usual chromoproteins.

I have analysed the ultra-violet portion of the spectrum by my procedure. The presence of two definite bands at 280 and 260 mμ, which closely overlap, explains the high extinction at 260 mμ, but does not fully account for the marked elevation of the γ-band, nor the more moderate elevation of the α- and β-bands.

It is perhaps possible to account for the maximum at 475 mμ by the absorption due to the flavin phosphate. I was hence left with two possible explanations. There was either some systematic error in the spectral measurements (probably unlikely), or indeed some modifying influence upon the haemin itself. Morton's proposal that the flavin nucleus is in the proximity of the haemin is appealing from the viewpoint of the modifying influence such a structure might have upon the absorption spectra of the iron-porphyrin complex.

O'HAGAN: I should like to draw attention to the spectra of a haemoprotein with the opposite type of spectra to that described by Morton, namely, one with a very low Soret peak. This may be seen in the curves for aetiomyoglobin and its CO- and ferri-derivatives described by O'Hagan and George (*Biochem. J.* **74**, 424, 1960). Mesohaemin and aetiohaemin in chloroform have essentially the same absorption spectrum, but when linked to apomyoglobin the meso spectrum is much more pronounced. It may be that in cytochrome b_2 a very strong linkage of the haem propionate groups to the apoprotein may contribute to the absorption.

GEORGE: Is it possible that direct co-ordination between flavin and haem iron (presumably through one of the nitrogen atoms) could conceivably play any part in cytochrome b_2 reactions?

MORTON: We have given some consideration to this type of compound. In such a complex, one might expect that the extinction coefficients of the principal absorption bands of the haemochrome would be elevated as compared with haemochromes formed with the flavin-free system.

As pointed out in our paper (Morton, Armstrong and Appleby, this volume, p. 510), we have observed a temporary shift of the α- and β-bands of ferrocytochrome b_2 by about 10 mμ towards longer wavelengths to occur under certain conditions. This suggests that there is some type of flavin-haem interaction when the 'lactate'-cytochrome b_2 complex has lost one electron, or: viz. a free radical is formed. This would support the idea that a nitrogen atom of the iso-alloxazine ring may be co-ordinated to the iron atom of the protohaem in cytochrome b_2.

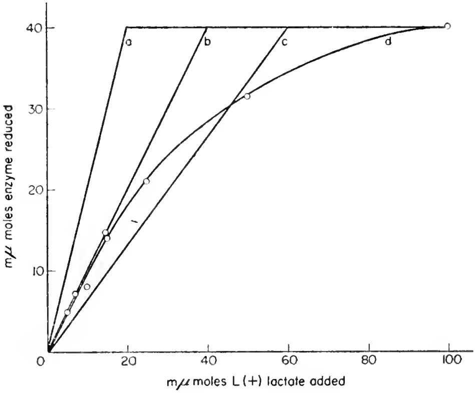
Of course, as suggested by Falk there may be steric restrictions.

It is apparent that there is a need for study of possible complexes of riboflavin and of related compounds with protohaem.

The Oxidation-reduction Changes in the Reaction of Lactate with Cytochrome b_2

By E. BOERI AND E. CUTOLO (Ferrara)

BOERI: One experiment which we performed seems to me important and I want to refer to it. We have titrated flavocytochrome b_2 with the substrate. The titrations were performed in both directions, by addition of ferricyanide to reduce flavocytochrome b_2 or by addition of L(+)-lactate to oxidized flavocytochrome b_2. The titrations were performed in anaerobic conditions, by following the extinction changes at 557 mμ in a Thunberg-shaped cuvette. In both instances, plots of the equivalents used versus the moles of enzyme reduced showed strict adherence to a ratio of 2 equivalents/mole for the first part of the curve, up to about 50% reduction and oxidation. The second part of the curve showed that to obtain complete reduction or oxidation more equivalents of the titrating agent were necessary. Our experiments were performed with about 50 millimicromoles of flavocytochromes b_2. In view of the 2:1 ratio obtained in these experiments, we favour a reaction of the type:



Abscissa: millimicromoles of L(+)-lactate added from the burette. Ordinate: reduction as judged from the increase at 557 mμ. Line *a:* theoretical for a stoichiometry lactate: enzyme = 1:2; line *b* for a stoichiometry 1:1, *c* for a stoichiometry 2:3; line *d* is the best fitting line.

Conditions of the experiment: pH 7·0, phosphate buffer 0·01 M, 20°C, *in vacuo*.

$$\text{Lactate} + \text{Prot} - \text{FMNH} - \text{cyt}^{+++} \rightarrow \text{Pyruvate} + \text{Prot} - \text{FMNH}_2 - \text{cyt}^{++}$$

in which the enzyme exchanges one electron on the haematin iron and one only on the flavin moiety. This hypothesis can account for the fact that the spectrum of oxidized flavocytochrome b_2 does not show the bands of oxidized flavin.

Experiments were begun, in co-operation with A. Ehrenberg of the Medical Nobel Institute of Stockholm, to detect the eventual presence of a free radical by electron spin resonance measurements.

The Function and Bonding of the Flavin Group of Cytochrome b$_2$

The Bonding between the Flavin Group and Apoprotein of Cytochrome b_2

By J. McD. ARMSTRONG, J. H. COATES AND R. K. MORTON (Adelaide)

MORTON: The studies reported here suggest that the riboflavin phosphate prosthetic group of cytochrome b_2 is bonded to the protein by thiol groups of cysteine residues. The linkage is extremely labile, and irreversible dissociation of the flavin may occur in the presence of oxygen or on addition of —SH binding reagents. Where flavin dissociation has occurred in the presence of oxygen, aggregation of the protein also occurs, presumably due to the formation of intermolecular disulphide bonds. This behaviour is not observed when flavin dissociation is brought about with *p*-chloromercuriphenylsulphonate (PCMS).

Unless otherwise indicated, these studies were carried out with freshly prepared twice crystallized cytochrome b_2 under anaerobic conditions; solutions were handled in an atmosphere of nitrogen at 2°C. The buffer used for most of the experiments had the following composition: 0·3 M sodium lactate, 0·05 M tetra sodium pyrophosphate/HCl, 10^{-4} M ethylenediamine tetra-acetate (EDTA), pH 6·85 ± 0·03, ionic strength 0·63. A few of the preliminary experiments were carried out in 0·4 M sodium lactate, 0·15 M sodium chloride, 10^{-4} M EDTA, pH 6·80, ionic strength 0·65. EDTA and high lactate concentrations protect cytochrome b_2 (Morton *et al.*, this volume, p. 501; Boeri and Rippa, this volume, p. 531). Pyrophosphate was chosen as the buffer ion because of its protective effect (Armstrong and Morton, unpublished). The rotor temperature was usually 2–5°C.

PROPERTIES OF INTACT CRYSTALLINE CYTOCHROME b_2 (DEOXYRIBONUCLEOPROTEIN)

In these buffers, cytochrome b_2 sediments as a single component with a symmetrical boundary, $s^0_{20,\mathrm{w}} = 8{\cdot}48 \times 10^{-13}$ sec. The concentration dependence of sedimentation, over the concentration range 8–110 μmoles haem/l., is described by the relationship $s^c_{20,\mathrm{w}} = s^0_{20,\mathrm{w}}\,(1 - 1{\cdot}54 \times 10^{-3}\,c)$ where c is expressed in terms of μmoles of enzyme haem/l. The pink colour of cytochrome b_2 may be observed to sediment with the boundary; in these experiments no detectable fluorescence of the solutions was observed before or after centrifugation. The lactate reductase activities towards ferricyanide and ferricytochrome *c* were typical of intact cytochrome b_2. The spectral properties of such preparations are: $\lambda_{\max}$ at 265·0, 330·0, 423·0, 527·5 and 556·5 mμ; and $E_{265\ m\mu}/E_{423\ m\mu}$, 0·89.

PROPERTIES OF NUCLEOTIDE-FREE CYTOCHROME b_2

The polydeoxyribonucleotide was dissociated from intact cytochrome b_2 by dialysis, under nitrogen, against ammonium sulphate solutions containing lactate and EDTA (see Appleby and Morton, *Biochem. J.* **75**, 258, 1960). Here also a single sedimenting component was observed, the pink colour again sedimenting with the boundary: $s^0_{20,\mathrm{w}} = 7{\cdot}87 \times 10^{-13}$ sec.

With adequate precautions, particularly with respect to oxygen and to possible contamination with copper, no fluorescence of the solutions can be detected before or after centrifugation. The concentration dependence (measured over the concentration range 8–103 μmoles haem/l.) may be described as follows $s^c_{20,\mathrm{w}} = s^0_{20,\mathrm{w}}\,(1 - 5{\cdot}33 \times 10^{-4}\,c)$ where the value of c is estimated in μmoles of haem/l. (based on the millimolar extinction coefficients of intact cytochrome b_2). Other properties of such preparations are: $\lambda_{\max}$ at 267–8, 328, 423·0, 528·0 and 556·5 mμ; and $E_{268\ m\mu}/E_{423\ m\mu}$, 0·6. Enzymic activities of such preparations are comparable with those of intact cytochrome b_2.

These results indicate that the polydeoxyribonucleotide has little influence on the sedimentation behaviour of the reduced native flavohaemoprotein. The polynucleotide contains approximately 15 nucleotide residues/protein-bound haem, corresponding to a weight of 5,000 g/mole of protein-bound haem; intact cytochrome b_2 has a weight of approximately 80,000 g/mole of protein-bound haem.

Preliminary studies of the polydeoxyribonucleotide dissociated from the intact crystalline enzyme by treatment with ammonium sulphate indicate that it behaves as a single compound when chromatographed on Ecteola resin. Since only a small quantity of material was available, the sedimentation behaviour is difficult to interpret, but no obvious heterogeneity was observed, and the slow rate of sedimentation indicates a low molecular weight (Montague and Morton, *Nature, Lond.* **187**, 916, 1960).

EFFECT OF OXYGEN ON REDUCED CYTOCHROME b_2

In the early experiments, oxygen was not rigorously excluded during the filling of the ultracentrifuge cell. In some cases it was observed that the material showed a yellow fluorescence and the solution, when examined with white light, appeared to have a brownish tinge when compared with freshly prepared cytochrome b_2. In the ultracentrifuge, two additional small boundaries appeared, leading the main boundary. For one such preparation, values of $s_{20,w}$ of 7·88, 11·22 and 14·54 × 10^{-13} sec were obtained. In addition, the solution to the centripetal side of the boundary appeared to be yellow.

It is evident that exposure of the enzyme to oxygen may cause (1) dissociation of some of the flavin, which becomes oxidized and therefore fluorescent, accompanied by (2) aggregation (polymerization) of some of the protein, presumably the material from which flavin has dissociated.

EFFECT OF TREATMENT OF CYTOCHROME b_2 WITH *p*-CHLOROMERCURIPHENYLSULPHONATE (PCMS)

Previous studies (Appleby and Morton, *Nature, Lond.* **173**, 749, 1954; Boeri and co-workers, *Arch. Biochem. Biophys.* **60**, 463, 1956) have shown that mercurials such as PCMS are powerful inhibitors of the lactate-ferricyanide and lactate-ferricytochrome *c* reductase activities of cytochrome b_2. On anaerobic incubation for about 15 min of intact, non-fluorescent cytochrome b_2 in the standard buffer with PCMS at a final concentration of 1 × 10^{-4} M, the solution showed an intense yellow fluorescence, while the colour changed from coral pink to a brownish pink. Spectroscopically the α- and β-bands of the reduced haemoprotein component appeared unaltered.

The material so treated sedimented in the ultracentrifuge as a single component, leaving a yellow non-sedimenting component behind. On exposure of the PCMS-treated material to air, the α- and β-bands of the reduced haemoprotein rapidly disappeared, showing that it was now readily autoxidizable. The oxidized material showed the same sedimentation behaviour as observed before oxidation. The sedimentation coefficient was not greatly altered from that of intact cytochrome b_2.

It is apparent that treatment with PCMS displaces the flavin prosthetic group, and at the same time prevents the aggregation which occurs in the presence of oxygen.

As already pointed out (Appleby and Morton, *Biochem. J.* **73**, 539, 1959), at alkaline or acid pH the flavin group is rapidly displaced with corresponding loss of enzymic acitivity. The reaction is catalysed greatly by copper ions (Morton *et al.*, this volume, p. 507), and the rate of autoxidation of cytochrome b_2 is strongly influenced by the ionic strength of the medium (Boeri and Rippa, this volume, p. 524).

Taken together with the results reported here, these observations suggest that the flavin group may be held by labile hydrogen bonds to thiol groups of cysteine residues of the protein. The reactivity of such thiol groups would expect to be greater at alkaline (> pH 8) as compared with neutral pH values. After displacement of the flavin, the thiol groups may be oxidized to —S—S— groups, thus forming intermolecular cross-links. When blocked by suitable compounds, the thiol groups are no longer capable of such reactions. This structure may explain the considerable lability of cytochrome b_2, and shows how modification of the native material may readily occur. There are eighteen ½-cystine groups/haem in cytochrome b_2 (see Morton, 1958, *loc. cit.*; Appleby, Morton and Simmonds, *Biochem. J.* **75**, 72, 1960).

The results reported here give further evidence that cytochrome b_2 is a single protein having flavin and haem prosthetic groups.

Although it is possible that the displacement of flavin and the observed aggregation are not directly related, the probable explanation of the observations is that the displacement of the flavin uncovers groups which are: (a) reactive with PCMS, (b) capable of oxidation to form aggregates, and (c) reactive with Cu^{++} ions. If thiol groups of cysteine residues in the protein are bonded to the flavin by hydrogen bonding (which would be influenced by changes of pH and ionic strength), this would provide a structure which would meet all the known experimental requirements. We believe that oxidation of the very reactive thiol groups by air suffices to explain the formation of aggregates.

Boundary analysis of the sedimentation of intact crystalline cytochrome b_2 shows that the spreading of the boundary with time can be accounted for wholly on the basis of diffusion, if this follows a Gaussian distribution (Baldwin, *Biochem. J.* **65**, 503, 1957).

Extensive studies of cytochrome b_2 and its modified forms by the approach to equilibrium method for the ultracentrifuge determination of molecular weight lead to the conclusion that under the conditions employed, cytochrome b_2 may behave as an interacting system, although nothing has been deduced as to the nature of the interaction.

MARGOLIASH: I should like to ask whether you have some evidence other than the reaction of *p*-chloromercuriphenylsulphonate for the idea that a sulphydryl group is an essential binding point of the flavin to the protein in yeast cytochrome b_2. *p*-Chloromercuriphenylsulphonate is not strictly specific for —SH and could possibly react with other amino acid side chains such as imidazole groups. Thiol groups in proteins also vary very considerably in their reactivity to so-called sulphydryl reagents. Did you study the kinetics and stoichiometry of the reaction either spectrophotometrically or by estimation of the protein-bound mercury? Was an independent titration curve for the —SH groups obtained? N-ethylmaleimide is considered a more specific reagent for —SH than the mercurials and would probably be safer in terms of interpretations of the mechanism of liberation of flavin from cytochrome b_2.

MORTON. The following evidence strongly suggests that thiol groups are involved in the binding of riboflavin phosphate to the protein.

1. Oxygen, Cu^{++} salts, H_2O_2 and *p*-chloromercuriphenylsulphonate (PCMS) all cause a loss of activity and associated fluorescence indicating a change in the bonding of the flavin prosthetic group.
2. Amino acid analyses and sulphur determinations suggest that there are about eighteen $\frac{1}{2}$-cystine residues/haem. We have found that only 4 —SH groups/haem react with PCMS, which completely displaces the flavin from the enzyme.

We have not, as yet, studied the effect of N-ethyl maleimide on the enzyme. However, Boeri and colleagues showed that iodoacetate is a poor inhibitor of cytochrome b_2 as compared with PCMS. This suggests that some of the thiol groups essential for activity are 'masked' by hydrogen bonding (or other type of bonding). Moreover, the formation of only two aggregates, by oxidation, rather than a multiplicity of aggregates, is evidence for the formation of —S—S— bonds at very specific sites of the protein (see Armstrong, Coates and Morton, *Nature, Lond.* **186**, 1033, 1960).

BOERI: In my experience the enzyme is sensitive to some inhibitors which act on —SH groups but not to others. The sensitivity to *p*-chloromercuribenzoate is high. The enzyme is less sensitive to *o*-iodosobenzoate. Monoiodoacetate does not inhibit at the usual pH of the test, but is inhibitory at pH 5 or lower. The enzyme is very sensitive at pH higher than 7 to sulphanilamide disulphides, as was shown by Brighenti. These compounds act by a reaction of the type:

$$\text{Enzyme—SH} + \text{RSSR} = \text{Enzyme—SSR} + \text{RSH}$$

Possible Free Radical Formation in Flavoproteins

By C. D. LUDWIG (Philadelphia)

LUDWIG: The comments which I have to make do not directly concern flavoprotein-haem interactions, but pyridine nucleotide-flavoprotein interactions. However, they might have some relevance, by analogy, to some of the findings of Morton, and Boeri and their collaborators, in their studies of cytochrome b_2. The work which I shall describe was done in Theorell's laboratory by Ehrenberg and myself.

Many of you may recall that Haas, more than twenty years ago, described a red complex of old yellow enzyme (OYE) produced by reducing the enzyme in the presence of excess triphosphopyridine nucleotide (TPN). Haas attributed the red complex which displayed a shift in the absorption band from 465 mμ to 475 mμ, to free radical formation, and this has frequently been cited as the best evidence for the formation of a semiquinone intermediate in a biological oxidation reaction. Recently Beinert ascribed to free radical formation a broad absorption band (550–660 mμ) with a peak at about 565 mμ, that occurred upon reduction and subsequent oxidation of a number of flavin enzymes, chiefly acyl dehydrogenases. This absorption band was accompanied by a change in colour from yellow to green-brown. With old yellow enzyme, his results were equivocal, yet in no instance did Beinert find a red complex or the spectral changes described by Haas.

While preparing OYE, one of us obtained a red compound with spectral properties similar to those described by Haas, when the protein was precipitated with alcohol at −5°C. Therefore we attempted to repeat Haas' experiment to determine by means of paramagnetic resonance absorption whether the red complex was a free radical. Under a variety of conditions we were able to get a red complex with shift of the two absorption peaks at 383 and 465 mμ to 392 and 475 mμ. This was produced at room temperature by addition of a 10–15-fold excess of TPNH to a solution of OYE in neutral phosphate buffer. The presence of the pyridine nucleotide in the reduced form appeared to be essential but exclusion of oxygen or addition of dithionite was not necessary. Despite careful search we could find no distinct peak at 565 mμ, but only a slight increase in absorbancy of the order that Beinert found with OYE. This appeared to be due to the shift of the large peak to 475 mμ. By ultracentrifugation of the red complex in a separation cell and enzymic assay of the TPN remaining uncombined in the supernatant, it was shown that the amount of TPN bound by the enzyme was equivalent to its FMN content—that is, 2 moles/mole of protein. Free radical formation was then demonstrated by paramagnetic resonance absorption, by using higher concentrations of OYE (240 μM to 1 μM), and TPNH. A very large ESR signal was obtained, which had a g-value of 2·002 indicating an organic free radical. The derivative curves were indistinguishable in shape, width and g-value from those obtained by reduction of FMN with zinc in acid solution. Our results indicated that the red complex contains TPN in addition to FMN, as Haas assumed. However, the red complex is not identical with the free radical, since independent measurements showed that free radicals comprised only about 15% of the total enzyme concentration (FMN equivalents). Free radicals appeared to accumulate until a steady-state was reached and disappeared when the solution was exposed to air.

Further studies suggested that the red complex contains TPN and FMN, both in the oxidized form. Thus, it appears to be, in effect, an oxidized ES complex, or an undissociated enzyme-product (EP).

One is reminded that complexing of FMN to the apoprotein to form OYE, shifts the two absorption peaks of the former (372, 450 mμ) to 383 and 465 mμ, respectively. We now have evidence that attachment of the pyridine nucleotide produces a further shift toward long wave lengths to 391 and 475 mμ, respectively. The shift of the peaks of cytochrome b_2 from 527 and 557 to 533 and 567, during an intermediate stage of oxidation, that Morton and co-workers have described, might have an analogous interpretation. The titration data of Boeri and colleagues suggest that a free radical is likely, and the paramagnetic resonance absorption studies which he has planned should afford a final answer to this.

Autoxidation of Cytochrome b$_2$

Margoliash: In connexion with the findings on the effect of ionic strength on the rate of autoxidation of reduced cytochrome b_2 may I point out that a rather similar situation occurs with cytochrome c. Although native mammalian heart cytochrome c has a very slow rate of autoxidation this rate can be increased quite considerably by increasing the ionic strength. Denatured cytochrome c shows a varying rate of autoxidation depending on the degree of denaturation and in this case too, the rate of autoxidation of each individual sample will be a function of the ionic strength of the medium.

WINFIELD: In regard to the question on rate of oxygen uptake by the enzyme, it seems quite possible that a small change in the mode of attachment of flavin to the protein, during isolation of the enzyme, could result in the flavin becoming autoxidizable, as indicated by Morton; vice versa, the capacity of the flavin moiety to combine with oxygen, if it is present in the native enzyme, could be lost during isolation.

WILLIAMS: The autoxidation of cytochrome b_2 is written by Morton and co-workers as an attack on the haem iron. The results of Boeri and colleagues indicate that the reaction is an attack on free iron. The experiments of Boeri and co-authors are very like those of Warburg on the catalysed oxidation of cysteine by haemochromes. Warburg takes the point of view of Morton. In a discussion of this reaction I have proposed that Warburg's mechanism is incorrect (Williams, *Chem. Rev.* **56**, 299, 1956), and I have cited evidence in favour of an attack essentially the same as Boeri's discussion of his reaction. Could Morton and Boeri comment on the differences in their observations which lead them to write the reaction differently?

MORTON: Oxidation of cytochrome b_2 apparently proceeds in several stages. The intact flavohaemoprotein has a very low activity with oxygen. We are in agreement with Boeri and his colleagues that water is the product of this reaction. This may involve a reaction of oxygen with the haem iron. Alternatively if the flavin of the intact reduced cytochrome exists as FMNH in 'Protein–FMNH–haem–Fe^{+++}' in oxidized cytochrome b_2 (see Boeri and Rippa, this volume, p. 524), an attack on the flavin is possible. Whatever the mechanism, some flavin prosthetic group becomes fluorescent, probably due to oxidation of thiol groups as suggested earlier (Morton, this volume, p. 571). The riboflavin phosphate which is no longer bonded to the protein as in native cytochrome b_2 possibly due to a bond between the flavin and haem having been broken, may now react directly with oxygen to form H_2O_2. Evidence for the formation of H_2O_2 during prolonged exposure to oxygen is provided by the protection obtained with catalase (see Morton and co-workers, this volume, p. 506). H_2O_2 may rapidly oxidize the labile thiol groups, causing further denaturation of the flavohaemoprotein, and dissociation of flavin.

Denatured cytochrome b_2 formed by disruption of the normal bonds between the flavin prosthetic groups and the protein (as, for example, by treatment of cytochrome b_2 with *p*-chloromercuriphenylsulphonate), is highly autoxidizable.

It would appear that the presence of the flavin group hinders attack of oxygen on the haem iron of intact cytochrome b_2. This *may* be due to the fact that the flavin is bonded directly to the haem iron. Alternatively, the presence of the flavin group is associated with folding of the polypeptide chains which restricts oxygen attack on the haem group; displacement of the flavin causes unfolding of the polypeptide chains and facilitates oxygen reaction with the haem. I consider that the reaction of denatured cytochrome b_2 with oxygen is probably analogous to the reaction of the denatured cytochrome *c* (at pH > 12) with oxygen.

WINFIELD: Is it known as to how the amount of cytochrome b_2 in yeast is affected by the oxygen tension during growth?

BOERI: The cytochrome b_2 is particularly abundant when the yeast is well aerated and in stationary conditions of growth. Anaerobic yeast lacks cytochrome b_2, but has instead other enzymes acting on lactate.

THE ROLE OF CYTOCHROME *b* IN THE RESPIRATORY CHAIN

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Cytochrome *b* was one of the three cytochromes whose bands were first seen with the microspectroscope by McMunn (1886) and Keilin (1925). In the reduced state, it shows strong absorption bands at 564 mμ (α), 530 mμ (β) and 432 mμ (γ). It is one of the five cytochromes known to be present in the mitochondria of animal tissues and is also present in many microorganisms. This distribution makes it highly probable that it is a component of the *main cytochrome system* which is involved in the main respiratory pathway of most aerobic cells. Nevertheless, we know less about this cytochrome than of many other cytochromes, including those in the *b* group (e.g. cytochrome b_2 or b_5). One of the reasons for this is that it has never been isolated in a purely soluble, and enzymically active form.

The first studies of Keilin (1925) showed that cytochrome *b* has some unique properties. When a suspension of yeast cells was aerated in the presence of urethane, the *b* band remained visible (i.e. cytochrome *b* was reduced), while the *a* and *c* bands disappeared (i.e. cytochromes *a* and *c* were oxidized). This indicated rather strongly that cytochrome *b* is the member of the chain which acts nearest the substrate. This was further supported by Ball's (1938) measurements of the oxidation-reduction potential of the three cytochromes, which showed that cytochrome *b* had a much lower potential (−40 mV at pH 7·4) than the other two (+260 mV and +290 mV, respectively). It has, perhaps, not always been sufficiently emphasized that the fact that cytochrome *b* acts nearer to the substrate than the other cytochromes implies that some kinetic differences between this cytochrome and the others are to be expected, and need not be abnormal.

Keilin and Hartree (1939) emphasized the close association of succinate dehydrogenase with cytochrome *b* and at one time (see, for example, Ball, Anfinsen and Cooper, 1947; Slater, 1949) the possibility that the two were identical was seriously considered, especially when it appeared from the studies of Slater (1950) that cytochrome *b* was not involved in the oxidation of reduced diphosphopyridine nucleotide (DPNH). However, Tsou (1951) showed that it was possible to inhibit succinate dehydrogenase (by incubation with cyanide), without decreasing the amount of cytochrome *b* reduced, clearly showing that the two were not identical, while at about the same time Morton (1950) obtained an active succinate dehydrogenase in a solution free from haematin compounds. Further purification of the dehydrogenase, particularly by Singer and Kearney (1954) and Wang, Tsou and Wang (1956), has shown quite conclusively that it does not contain a haematin.

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Early observations with the microspectroscope indicated that when succinate was added to a respiratory-chain preparation (e.g. the Keilin and Hartree heart-muscle preparation) saturated with air, the *b* band rapidly appeared as soon as oxygen was exhausted from the suspension (about 2 sec). With the eye, cytochrome *b* in active preparations was reduced as rapidly as cytochromes *a* and *c*, and there was no reason to suspect that it did not lie on the main pathway between succinate and oxygen. The same result was obtained when succinate was added to the preparation in the absence of oxygen, or in the presence of oxygen and cyanide. On the other hand, DPNH, under these conditions, reduced cytochrome *b* so much more slowly that it was concluded that cytochrome *b* was on a side path for the oxidation of DPNH (Slater, 1950).

In 1952, Chance began his important measurements of the rate of reduction of the components of the respiratory chain, first in the Keilin and Hartree heart-muscle preparation, later in mitochondria. His conclusions concerning the role of cytochrome *b* in the respiratory chain were:

(1) In heart-muscle preparations, cytochrome *b* is reduced by succinate at a rate which is inconsistent with a place for this cytochrome in the main pathway (Chance, 1952).

(2) In intact yeast cells (Chance, 1954), or in isolated rat-liver mitochondria (Chance, 1955), cytochrome *b* is reduced rapidly not only by succinate, but also by diphosphopyridine nucleotide (DPN)-requiring substrates. Chance and Williams (1955) concluded that when oxidative phosphorylation takes place, cytochrome *b* is in the main pathway for both substrates, and that the non-phosphorylating systems are artefacts, in which cytochrome *b* has lost its capacity to participate in the respiratory chain.

(3) The addition of antimycin (or 2-n-heptyl-4-hydroxyquinoline N-oxide) to a heart-muscle preparation causes an increase in the rate of reduction of cytochrome *b* by DPNH (Jackson and Lightbown, 1958) and by succinate (Chance, 1958), and in the amount of cytochrome *b* (or similar pigment) reducible by succinate (Chance, 1958). Chance concluded that antimycin alters the system in such a way that the electrons that flow to cytochrome c_1 in its absence are routed to cytochrome *b* in its presence.

The remainder of this paper will be devoted to a description of our recent experiments relevant to the problem of the role of cytochrome *b*, and to the tentative conclusions which we draw from the results obtained by ourselves, and by Chance.

PIGMENTS PRESENT IN HEART-MUSCLE PREPARATION, WHICH ABSORB IN THE SAME REGION OF THE SPECTRUM AS CYTOCHROME *b*

Oxymyoglobin and Myoglobin

Colpa-Boonstra and Minnaert (1959) have shown that even carefully washed heart-muscle preparations contain sufficient oxymyoglobin to cause interference in measurements of cytochrome *b*. This pigment was identified by an accurate determination of the difference spectrum obtained by the addition of CO to a deoxygenated heart-muscle preparation (Fig. 1). The peak in the difference spectrum was at 422 mμ, which showed that the pigment was myoglobin and not haemoglobin, the CO compound of which absorbs at 419·5 mμ in the difference spectrum. This conclusion was confirmed by adding myoglobin and haemoglobin. The addition of the first pigment did not cause any shift in the position of the band, but this moved towards a

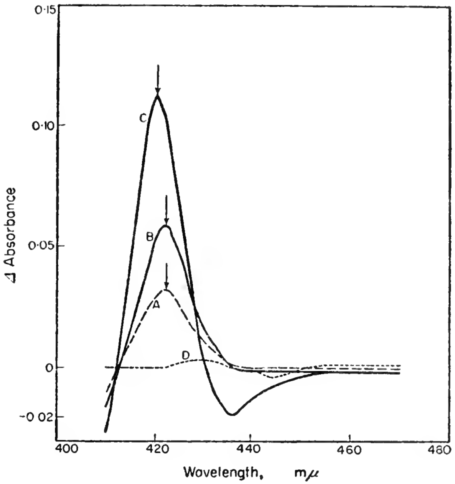

FIG. 1. Demonstration of myoglobin in heart-muscle preparation. Difference spectra: (CO-compounds *minus* deoxygenated). *A*, normal heart-muscle preparation; *B*, *idem*, after tipping in myoglobin; *C*, *idem*, after tipping in haemoglobin; *D*, heart-muscle preparation treated with $NaNO_2$. Light path, 1 cm. Normal heart-muscle preparation, 1·16 mg protein/ml; $NaNO_2$-treated preparation, 1·56 mg protein/ml. (From Colpa-Boonstra and Minnaert, 1959.)

shorter wavelength when haemoglobin was added. Under the conditions of these measurements, cytochrome a_3 does not interfere, since it remains in the oxidized state and does not combine with CO.

The amount of oxymyoglobin present was 0·2–0·3 μmole/g protein (cf. cytochromes $c + c_1 +$ protohaematin compounds, about 1·5 μmoles/g protein).

Oxymyoglobin interferes in measurements of the difference spectra (reduced *minus* oxidized) when succinate is substrate, since it is deoxygenated to myoglobin when the suspension goes anaerobic. This can be eliminated by previous treatment of the preparation with nitrite to oxidize the oxymyoglobin to metmyoglobin, as suggested by Chance (1952). Metmyoglobin is not reduced by succinate in the presence of the heart-muscle preparation.

Other Cytochromes

Although the absorption bands of the cytochromes are sharp, they are sufficiently close together to cause considerable overlapping. It is desirable, therefore, to choose a wavelength where changes of absorbancy are due only to cytochrome *b*, and not to the other cytochromes. Holton (1955) has introduced a method, which enables this to be determined. In heart-muscle preparation oxidizing succinate in the presence of air, the cytochromes are largely oxidized, and become reduced when the suspension becomes anaerobic (Chance, 1952). Holton (1955) found that, in the presence of malonate, the reduction of cytochrome *b* so lagged behind that of the other cytochromes that in the region of the peaks of this cytochrome the first change was a decrease of absorbancy caused by reduction of the other cytochromes, followed by an increase of absorbancy caused by reduction of cytochrome *b*. By carrying out the measurements at different wavelengths, it was possible accurately to determine the wavelength at which the first decrease of absorbancy did not occur. This represents the isosbestic points of all the other cytochromes taken together. These were found to be at 434 mμ and 558 mμ (Colpa-Boonstra and Holton, 1959).

Chance (1958) introduced another method of eliminating the contribution of the other cytochromes to the spectrum. This makes use of the fact that, in the presence of cyanide, ascorbate reduces all the cytochromes except *b*. This method is very suitable for accurately determining the spectrum obtained by addition of succinate, and by the subsequent addition of antimycin. Neither spectrum obtained is influenced by the presence of oxymyoglobin or metmyoglobin, because the latter is not reduced and the former remains oxygenated since oxygen is not consumed by the solution in the presence of cyanide. However, we do not agree with Chance that the spectrum obtained by subsequent addition of $Na_2S_2O_4$ is not affected by the presence of these compounds; indeed, both would be reduced to myoglobin. This would be expected to lead to increased absorption in the regions 435 mμ and 556–566 mμ, which Chance ascribes to a cytochrome *b*-like pigment which is not reduced by succinate, even in the presence of antimycin.

OXIDATION-REDUCTION POTENTIAL OF CYTOCHROME *b*

Ball (1938) calculated the oxidation-reduction potential of cytochrome *b* from measurements with the visual spectroscope of the proportion of the total cytochrome *b* (measured with $Na_2S_2O_4$) which was reduced in the presence of mixtures of succinate and fumarate. He found a value of −40 mV at pH 7·4.

Slater (1953) drew attention to the fact that in Chance's (1952) first experiments, the [fumarate]/[succinate] ratio at the end of the experiment was 0·15, which would be expected to give only 37% reduction of the cytochrome *b*, if Ball's (1938) value for the oxidation-reduction potential of cytochrome *b* were correct. Since it was clear from Chance's data that the reduction had proceeded much further than this, Slater (1953) suggested that Ball's value might have to be raised (see Note 1).

Hill (1954) reported unpublished experiments suggesting that the oxidation-reduction potential of cytochrome *b* was in the region of zero.

The potential has been recently re-measured by Colpa-Boonstra and Holton (1959) by determination of the equilibrium between cytochrome *b* and succinate/fumarate in heart-muscle preparations from horse and pig. Absorbancy changes were measured spectrophotometrically in Holton's (1957) instrument at 434 mμ and 558 mμ, the isosbestic points of the other cytochromes taken together (see above). Interference by myoglobin was eliminated by treating the particles with nitrite.

The results were calculated by rearranging the equation for the equilibrium constant

$$K = \frac{[\text{fumarate}]}{[\text{succinate}]} \cdot \frac{[b^{++}]^2}{[b^{+++}]^2}$$

to give

$$\frac{1}{[b^{++}]} = \frac{1}{[b]} + \frac{1}{[b]\sqrt{K}}\sqrt{\frac{[\text{fumarate}]}{[\text{succinate}]}}$$

where $[b^{++}]$ and $[b^{+++}]$ are the concentrations of ferro- and ferricytochrome *b* respectively, and

$$[b] = [b^{++}] + [b^{+++}].$$

Values of K were calculated from the straight line (see Note 2) obtained by plotting $1/\Delta A$ against $\sqrt{\frac{[\text{fumarate}]}{[\text{succinate}]}}$, where ΔA refers to the observed absorbancy change associated with the reduction of cytochrome *b* from its oxidized state to its anaerobic equilibrium with succinate/fumarate. This method does not require the determination of the total cytochrome *b* content. The redox potential of cytochrome *b* was calculated from these values of *K* and the potential of the succinate/fumarate system, +24 mV at pH 7, 25°C (Borsook and Schott, 1931). The results ranged from +60 mV to +90 mV, with a mean value of +77 mV. These values obtained both from the γ- and the α-bands agree very closely with the data for the α-region published by Chance (1958), which would give an oxidation-reduction potential of 73–76 mV. However, Chance (1958) does not calculate a potential, because the directly determined equilibrium constant (42–54) did not agree with the ratio (10) of the second-order reaction constants for the forward and back reactions. It seems to us that when the reduction has reached a steady state in the absence of oxygen (when there can be no net electron transfer) the system has reached equilibrium: and that the disagreement between the two values must be ascribed either to experimental errors, or to the fact that the full expression for the equilibrium constant probably contains rate constants, which are not included in the bimolecular rate constants because they are not rate-limiting. The reduction of cytochrome *b* by succinate is perhaps rather complicated* (see below).

The difference between the 77 mV found by Colpa-Boonstra and Holton (1959) and the −40 mV of Ball (1938) is partly accounted for by the fact that the latter assumed that all the cytochrome *b* reducible by $Na_2S_2O_4$ could also be reduced by succinate. This is not the case (Chance, 1952, 1958; also, see below).

KINETICS OF THE REDUCTION OF CYTOCHROME *b*

We have measured the kinetics of the reduction of cytochrome *b* in the heart-muscle preparation by the two methods introduced by Chance (1952); (i) by following the process of reduction from the aerobic steady state to the anaerobic state while oxygen is consumed in the oxidation of succinate or DPNH; (ii) by measuring the rate of reduction by succinate in the presence of cyanide.

Figure 2, in which the recorder traces obtained with Holton's (1957) instrument have been converted into absorbancy changes, shows that the reduction of cytochrome *b* (measured at 432 mμ) and of cytochrome a_3 (at 448 mμ) by succinate or by DPNH, as the system proceeds from the aerobic steady state to the anaerobic state, are closely synchronized. Cytochrome *b* was followed at 432 mμ, because at this wavelength reduction of this cytochrome results in a large increase in absorbancy, while reduction of the other cytochromes causes a decrease. If the reduction of cytochrome *b* occurred much later than that of cytochrome a_3 this would be revealed by a small decrease of the absorption at 432 mμ, at the same time as the rapid increase at 448 mμ. This did not occur, unless inhibitors of the chain between succinate and cytochrome *b* (e.g. malonate) were added (cf. Holton, 1955). A comparison of the traces of cytochromes *b* and a_3 in Fig. 2 showed that reduction of cytochrome *b* began about 0·25 sec after the beginning of reduction of cytochrome a_3. This sequence would be expected if cytochrome *b* acted nearer substrate than cytochrome a_3.

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* *Added in proof.* Another explanation of this discrepancy has been given in the full paper by Holton and Colpa-Boonstra (*Biochem. J.* **76**, 179, 1960).

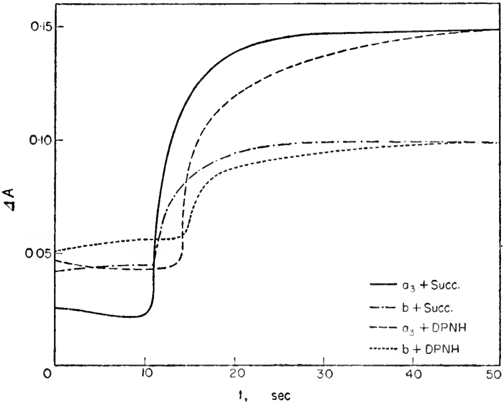


FIG. 2. Reduction of cytochromes *b* and a_3 in heart-muscle preparation by succinate or DPNH from the aerobic steady state to anaerobic steady state. Changes of absorbancy were calculated from the traces obtained at 432 mμ and 448 mμ with Holton's (1957) recorder. The position of the curves on the ordinate is arbitrary. Light path, 0·5 cm. Substrate (5 mM succinate or 1·5 mM DPNH) added at zero time to a suspension of $NaNO_2$-treated heart-muscle preparation (4·35 mg protein/ml) in 0·04 M phosphate, pH 7·4, 0·001 M ethylenediaminetetraacetate. The Q_{O_2} (μl. O_2/mg protein/hr) at 25° of the preparation was 368 for DPNH and 351 for succinate.

In Fig. 3, the reaction rates at different points of the absorbancy curve, divided by the difference in absorbancy between each point and the maximum, have been plotted against time. This quantity $dA/dt \, . \, 1/\Delta A$ has a formal relationship to a first-order reaction constant, with dimensions sec^{-1}, and has been designated k'_b and k'_{a_3} for the two cytochromes. The *k*'s are not constant, but increase from zero during the aerobic steady state to a maximum during the initial stages of the reduction, and decrease sharply again towards the end of the reaction. Although the actual values have no real meaning, being the result of measuring the difference between a reduction and an oxidation reaction, it appears significant that, both with succinate and with DPNH as substrate, k'_b and k'_{a_3} follow similar curves during the final stages of the reduction. We would not expect this behaviour if cytochrome *b* were not on the main pathway of reduction of cytochrome a_3, as suggested for DPNH by Slater (1950), and for succinate by Chance (1952).

The rapid reduction of cytochrome *b* by DPNH was in conflict with Slater's (1950) observations with the microspectroscope. On re-examination of this point, it was found to be a question of the concentration of DPNH. The relatively low concentration of DPNH (sufficient to saturate the DPNH oxidase system) used in the previous experiments was found to reduce the cytochrome *b* slowly and incompletely. The higher concentration (1·5 mM) used in the experiment shown in Fig. 2 was seen in the microspectroscope rapidly to reduce the cytochrome *b* (see Slater, 1958). Similar results have been reported by Jackson and Lightbown (1958).

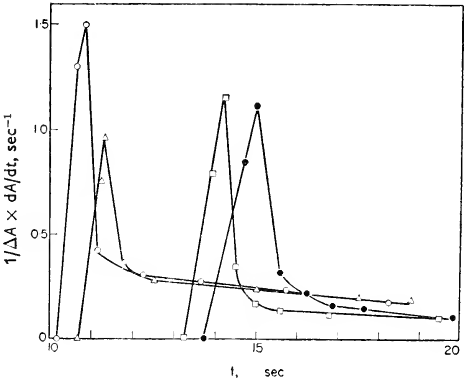


FIG. 3. The rates of reduction of cytochromes *b* and a_3, expressed as $1/\Delta A \cdot dA/dt$, calculated from Fig. 2, and plotted as a function of time. ○—○, a_3, succinate; △—△, *b*, succinate; □—□, a_3, DPNH; ●—●, *b*, DPNH.

In agreement with Chance's (1952) observations, it was found that the reduction of cytochrome *b* by succinate in the presence of cyanide was much slower than that calculated from the rate of the oxidation of succinate in the absence of cyanide, on the assumption that cytochrome *b* were on the main pathway. However, we think it possible that cyanide interferes with the reduction of cytochrome *b*, and that this slow rate of reduction is not characteristic of the uninhibited system (cf. Slater, 1958). In our view, the evidence is consistent with the proposition that cytochrome *b* is in the main pathway for succinate, and for high concentrations of DPNH. Mechanisms incorporating this view will be suggested later in the paper.

EFFECT OF ANTIMYCIN ON CYTOCHROME *b*

In agreement with the observations of Chance (1958), it was found that when antimycin was added to a heart-muscle preparation already reduced by succinate, the γ-band of cytochrome *b* was increased in intensity by 50–100% (Fig. 4). The higher absorption was also found when antimycin was added before the succinate.

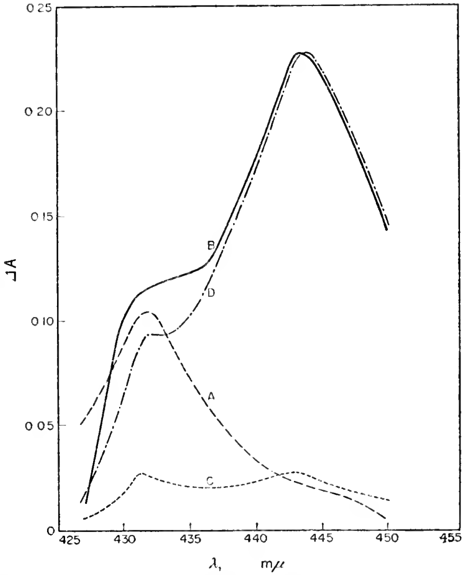


FIG. 4. Difference spectra of $NaNO_2$-treated heart-muscle preparation (5·58 mg protein/ml) reduced by succinate. Curve *C*, aerobic steady state *minus* oxidized, showing partial reduction of cytochromes in the aerobic steady state. Curve *A*, same as *C* in the presence of antimycin (0·09 μg/mg protein). Curve *D*, anaerobic steady state *minus* oxidized. Curve *B*, same as *D* in the presence of antimycin. Curves *A* and *C* are partially corrected for light scattering by subtraction of the absorbancy of *C* at 425 mμ (isosbestic point of reduced *minus* oxidized—Chance (1958)) and Curves *B* and *D* by subtraction of the absorbancy of *D* at 425 mμ. Light path, 0·5 cm. Suspension medium as in Fig. 2. The inhibition by the antimycin of the succinate oxidase activity was 31%.

In agreement with Chance (1958), the position of the peak of the γ-band of the difference spectrum (succinate + antimycin *minus* succinate) was at a slightly longer wavelength (432 mμ) than the band obtained with succinate alone (431 mμ).

As already mentioned, the extra absorption found by Chance (1958) at 435 mμ and 556–566 mμ by the addition of $Na_2S_2O_4$ to a system already containing succinate and antimycin can probably be ascribed to transformation of metmyoglobin and oxymyoglobin to myoglobin. It is our view that the data of Chance and ourselves provide evidence for the presence of two, not three, cytochrome *b*-like pigments. One of these (hereinafter referred to as cytochrome *b*) is reduced by succinate alone, or by $Na_2S_2O_4$. The other (referred to as cytochrome *b'*) is likewise reduced by $Na_2S_2O_4$, but is reduced by succinate only in the presence of antimycin.

Chance (1958) provides further data on the effect of antimycin which are very important for an understanding of the mechanism of its action. These measurements show that the addition of antimycin increases 50-fold the velocity of reduction of ferricytochrome *b* by succinate in the presence of cyanide and decreases 20-fold the velocity of oxidation of ferrocytochrome *b* by fumarate, also in the presence of cyanide.

Chance (1952) originally attributed the additional absorption obtained with antimycin to a combination of the latter with cytochrome *b*, or with a factor operating between cytochromes *b* and *c*. In his most recent article, Chance (1958) abandons this interpretation, because the intensified absorption was found with mitochondria, fly sarcosomes and other preparations. Instead, he suggests that the additional absorption resulting from the antimycin treatment is due to an inactive form of cytochrome *b*, i.e. one that can be activated only by the more rapid electron transfer to cytochrome *b*, which occurs in the presence of antimycin.

We agree that the increased absorption is due to reduction of a form of cytochrome *b* which is inactive in the absence of antimycin. However, the fact that antimycin both stimulates the reduction of active cytochrome *b* by succinate in the presence of cyanide, and inhibits its oxidation by the respiratory chain, suggests to us rather strongly that antimycin reacts also with the active cytochrome. We should like to suggest that antimycin combines with both cytochrome *b* and cytochrome *b'*, leading to an increased oxidation-reduction potential and thereby facilitating the reduction of both forms of cytochrome *b* by succinate. From the equilibrium constant determined by Chance (1958) in the presence of antimycin, it is possible to calculate that, under these circumstances, the E_0' is about 100 mV. However, this probably represents a value that lies somewhere between those for the antimycin compounds of the two forms of cytochrome *b*. It is possible that the reason for the failure of cytochrome *b'* to react with succinate is that, during preparation of the heart-muscle preparation, its structure is altered in such a way that its oxidation-reduction potential is decreased to a value well below that of succinate-fumarate.

There are two possible explanations for the fact that the addition of antimycin to a heart-muscle preparation reduced with succinate causes a displacement of the α- and γ-bands to a higher wavelength: (i) the bands of cytochrome *b'* are at a higher wavelength than those of cytochrome *b*; (ii) combination of both forms of cytochrome *b* with antimycin causes a displacement of the band towards longer wavelength.

Deul (1959) has made two observations which are of interest in connection with the possible relationship between the antimycin-sensitive site in the respiratory chain, and the 2:3-dimercaptopropanol (BAL)-sensitive site (Slater, 1949). In the first place, it was found that treatment of the heart-muscle preparation with BAL, in the presence of air, caused the destruction of cytochrome *b*′, and therefore abolished the extra absorption obtained by the addition of antimycin, in the presence of succinate. However, it is not yet known whether this destruction of cytochrome *b*′ is related to the inhibition of the respiratory chain brought about by incubation with BAL, or is a side reaction.

The second observation was that, although cytochrome *b* was readily reduced by succinate after BAL treatment, and by succinate or DPNH after antimycin treatment, the addition of antimycin to a BAL-treated preparation prevented the reduction of cytochrome *b* by succinate or DPNH. This observation supports other indications that, although the sites of action of antimycin and of BAL are closely connected, they are not identical.

POSSIBLE MECHANISM OF ACTION OF CYTOCHROME *b* IN THE RESPIRATORY CHAIN

To conclude this paper, we should like to suggest a mechanism, which is treated in greater detail elsewhere (Colpa-Boonstra, 1959), for the function of cytochrome *b* in the respiratory chain, which is based on the following data:

(1) In phosphorylating preparations of the respiratory chain, cytochrome *b* is rapidly reduced by added succinate or by endogenous DPNH.

(2) In non-phosphorylating preparations, cytochrome *b* is rapidly reduced by succinate, or by high concentrations of DPNH, but only slowly by low concentrations of DPNH.

(3) Cyanide inhibits the reduction of cytochrome *b* by succinate without affecting the reduction of the other cytochromes.

(4) In phosphorylating preparations, it is probable that one molecule of adenosine triphosphate (ATP) is synthesized for each pair of hydrogen atoms (or electrons) transferred from DPNH to cytochrome *b*, and also one for each pair of electrons transferred from cytochrome *b* to cytochrome *c*.

The fact that phosphorylation is coupled with the respiratory chain shows that the latter is much more complicated than merely a succession of hydrogen- or electron-transferring reactions. Moreover, we think that it is important to keep in mind that although the oxidation of the iron atom of cytochrome *b* by the iron atom of cytochrome *c* is a single-electron reaction, one molecule of ATP is synthesized for each two electrons transferred.

For some time now, we have been formulating oxidative phosphorylation by the type of mechanism shown in reactions (1)–(3b)

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2 \tag{1}$$

$$A \sim I + X \rightleftharpoons X \sim I + A \tag{2}$$

$$X \sim I + P_i \rightleftharpoons X \sim P + I \tag{3a}$$

$$X \sim P + ADP \rightleftharpoons X + ATP \tag{3b}$$

Reaction (1) describes a hydrogen transfer from AH_2 to B, in which the energy available from the oxido-reduction is retained in the energy-rich compound $A \sim I$. Reactions (2)–(3b) describe energy-transferring reactions leading eventually to the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i). I and X stand for unknown compounds of the system, which are necessary for the conversion of oxidation energy to phosphorylation, but which do not themselves undergo oxidation and reduction. The different I's required for the first two phosphorylation steps of the respiratory chain are indicated by I_1 and I_2.

Some recent experiments which have been described elsewhere (Slater, 1959) have brought the possibility to the fore that an oxidation is also necessary for the transfer reaction (2), and that I takes part in reaction (1) in a reduced form, and is oxidized in reaction (2). Slightly modifying the details of the reaction mechanisms proposed recently (Slater, 1959), we should like to suggest the following mechanisms for the phosphorylating steps between DPNH and cytochrome *b*, and between cytochrome *b* and cytochrome *c*, respectively.

The first phosphorylation step is described by reactions (4)–(7)

$$DPNH + fp + I_1H_2 \rightleftharpoons DPN \sim I_1H + fpH_2 \tag{4}$$

$$DPN \sim I_1H + 2b^{+++} - I_2 + X \rightleftharpoons DPN^+ + 2b^{++} - I_2 + H^+ + X \sim I_1 \tag{5}$$

$$X \sim I_1 + P_1 \rightleftharpoons X \sim P + I_i \tag{6}$$

$$X \sim P + ADP \rightleftharpoons X + ATP \tag{3b}$$

$$fpH_2 + I_1 \rightleftharpoons fp + I_1H_2 \tag{7}$$

$$\textit{Sum}\ DPNH + 2b^{+++} - I_2 + P_i + ADP$$
$$\rightleftharpoons DPN^+ + 2b^{++} - I_2 + H^+ + ATP$$

Reaction (4) is the same as reaction (1) for the specific case where AH_2 and B are DPNH and fp, respectively, while I is written in the form IH_2. Reaction (5) describes the oxidation of I_1H_2 (bound to the DPN) by cytochrome *b*, which is shown here already bound to I_2. It is possible that I_2 is a part of the cytochrome *b* molecule (e.g. a vinyl side chain of the porphyrin ring). Simultaneous with the reduction of cytochrome *b* by I_1H_2 in reaction (5), there is a transfer of I_1 to X. Reaction (6) is the same as (3a). Reaction (7) postulates the reduction of I_1 by the reduced flavoprotein formed in reaction (4). It should be noted that cytochrome *b* is reduced by I_1H_2, and I_1 by flavoprotein.

The second step is described in reactions (8)–(12)

$$b^{++} - I_2 + c_1^{+++} \rightleftharpoons b^{+++} - I_2 + c_1^{++} \tag{8}$$

$$b^{+++} - I_2 + c^{+++} + X \rightleftharpoons b^{+++} - I_2^{+} \sim X + c^{++} \tag{9}$$

$$b^{+++} - I_2^{+} \sim X + P_i \rightleftharpoons b^{+++} - I_2^{+} + X \sim P \tag{10}$$

$$X \sim P + ADP \rightleftharpoons X + ATP \tag{3b}$$

$$c_1^{++} + c^{+++} \rightleftharpoons c_1^{+++} + c^{++} \tag{11}$$

$$b^{++} - I_2 + b^{+++} - I_2^{+} \rightleftharpoons 2b^{+++} - I_2 \tag{12}$$

$$\textit{Sum}\ 2b^{++} - I_2 + 2c^{+++} + P_i + ADP \rightleftharpoons 2b^{+++} - I_2 + 2c^{++} + ATP$$

Reaction (9) is analogous to reaction (5), but I_2, unlike I_1, remains bound to (or is a part of) cytochrome *b* throughout the reaction. In this scheme, the iron atom of cytochrome *b* is oxidized by either cytochrome c_1 (reaction (8)) or by I_2^{+} (reaction (12)). I_2 is oxidized by cytochrome *c*, and is reduced by cytochrome *b*.

It is important to note that a fully oxidized preparation in the absence of substrate would be expected to have all its cytochrome *b* in its fully oxidized form $b^{+++} - I_2^{+}$ which is not shown to react in reactions (4)–(7). It is, therefore, necessary to postulate an initiating reaction, e.g. the reduction of the I_2^{+} by fpH_2

$$fpH_2 + b^{+++} - I_2^{+} \rightleftharpoons b^{+++} - I_2 + fpH + H^{+} \tag{13}$$

Once the reaction was initiated, it would proceed with increasing speed since each molecule of $b^{++} - I_2$ formed by reactions (4)–(7) from the $b^{+++} - I_2$ formed in reaction (13) would produce two molecules of $b^{+++} - I_2$ by reaction (12). Indeed, this could be the explanation of the marked delay in reaching a steady rate of oxidation when DPNH is mixed with heart-muscle preparation (Slater, 1950).

It seems likely that the loss of phosphorylating activity in the Keilin and Hartree heart-muscle preparation is a consequence of the susceptibility of 'energy-rich' intermediates to hydrolysis. Thus, it would be expected that the $DPN \sim I_1H$ formed in reaction (4) would be hydrolysed

$$DPN \sim I_1H + H^{+} \rightarrow DPN^{+} + I_1H_2 \tag{14}$$

and no $DPN \sim I_1H$ would be available to reduce cytochrome *b*. We suggest that, under these circumstances, the reaction follows the following course

$$DPNH + fp + I_1H_2 \rightleftharpoons DPN \sim I_1H + fpH_2 \tag{4}$$

$$DPN \sim I_1H + H^{+} \rightarrow DPN^{+} + I_1H_2 \tag{14}$$

$$fpH_2 + b^{+++} - I_2^{+} \rightleftharpoons b^{+++} - I_2 + fpH + H^{+} \tag{13}$$

$$\mathrm{fpH} + c_1^{+++} \rightleftharpoons \mathrm{fp} + c_1^{++} + \mathrm{H}^+ \tag{15}$$

$$c_1^{++} + c^{+++} \rightleftharpoons c_1^{+++} + c^{++} \tag{11}$$

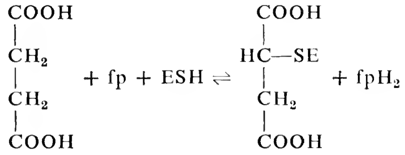
$$b^{+++} - \mathrm{I}_2 + c^{+++} + \mathrm{X} \rightleftharpoons b^{+++} - \mathrm{I}_2^{+} \sim \mathrm{X} + c^{++} \tag{9}$$

$$b^{+++} - \mathrm{I}_2^{+} \sim \mathrm{X} \xrightarrow{(\mathrm{H_2O})} b^{+++} - \mathrm{I}_2^{+} + \mathrm{X} \tag{16}$$

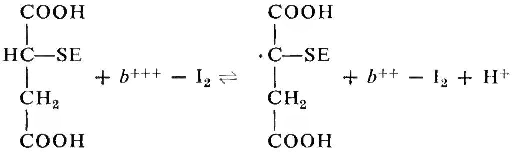
$$\textit{Sum}\ \mathrm{DPNH} + 2c^{+++} \rightarrow \mathrm{DPN}^+ + 2c^{++} + \mathrm{H}^+$$

The slow and incomplete reduction of cytochrome *b* often observed with heart-muscle preparation and DPNH could be due to dismutation of $b^{+++} - \mathrm{I}_2$ (back reaction (12)). Higher concentrations of DPNH might perhaps be expected to increase the rate of formation of $\mathrm{DPN} \sim \mathrm{I}_1\mathrm{H}$ to a point where sufficient survives hydrolysis to bring about reduction of cytochrome *b* by reaction (5) (see Note 3).

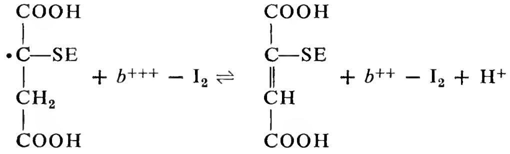
Although the reduction of cytochrome *b* by succinate can be adequately described by the sequence $\mathrm{SH}_2 \rightarrow \mathrm{fp} \rightarrow 2b^{+++}$, where the fp is succinate dehydrogenase, we should also like to bring forth the possibility that it proceeds by a mechanism similar to that suggested above for the reduction of cytochrome *b* by DPNH, with the exception that no labile energy-rich intermediates are formed. Bringing the —SH group of succinate dehydrogenase into the reaction, we could write



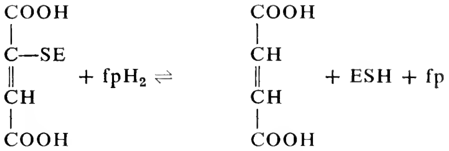
$$\mathrm{HOOC{-}CH_2{-}CH_2{-}COOH} + \mathrm{fp} + \mathrm{ESH} \rightleftharpoons \mathrm{HOOC{-}CH(SE){-}CH_2{-}COOH} + \mathrm{fpH_2} \tag{17}$$



$$\mathrm{HOOC{-}CH(SE){-}CH_2{-}COOH} + b^{+++} - \mathrm{I}_2 \rightleftharpoons \mathrm{HOOC{-}\dot{C}(SE){-}CH_2{-}COOH} + b^{++} - \mathrm{I}_2 + \mathrm{H}^+ \tag{18}$$

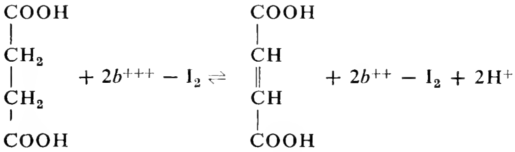


$$\mathrm{HOOC{-}\dot{C}(SE){-}CH_2{-}COOH} + b^{+++} - \mathrm{I}_2 \rightleftharpoons \mathrm{HOOC{-}C(SE){=}CH{-}COOH} + b^{++} - \mathrm{I}_2 + \mathrm{H}^+ \tag{19}$$



$$\begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{C{-}SE}\\ \|\\ \mathrm{CH}\\ |\\ \mathrm{COOH}\end{array} + \mathrm{fpH_2} \rightleftharpoons \begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CH}\\ \|\\ \mathrm{CH}\\ |\\ \mathrm{COOH}\end{array} + \mathrm{ESH} + \mathrm{fp} \qquad (20)$$

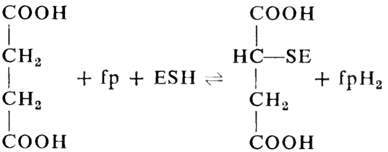
Sum



$$\begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{COOH}\end{array} + 2b^{+++} - \mathrm{I_2} \rightleftharpoons \begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CH}\\ \|\\ \mathrm{CH}\\ |\\ \mathrm{COOH}\end{array} + 2b^{++} - \mathrm{I_2} + 2\mathrm{H}^+$$

(It should be understood that fp and ESH are part of the same protein.)

Since there are no labile 'energy-rich' intermediates which would be subjected to hydrolysis in the non-phosphorylating preparations, the reduction of cytochrome *b* by succinate would also proceed rapidly in these preparations. It is possible that cyanide reacts with $b^{+++} - \mathrm{I_2}$ to give a product which can be oxidized back to $b^{+++} - \mathrm{I_2}^+$ by c^{+++} but whose iron atom cannot be reduced to ferrous. The oxidation can then proceed only by electron transfer through the $\mathrm{I_2}$ part of the $b^{+++} - \mathrm{I_2}^+$, following a path analogous to the non-phosphorylating pathway for DPNH, thus



$$\begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{COOH}\end{array} + \mathrm{fp} + \mathrm{ESH} \rightleftharpoons \begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{HC{-}SE}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{COOH}\end{array} + \mathrm{fpH_2} \qquad (17)$$

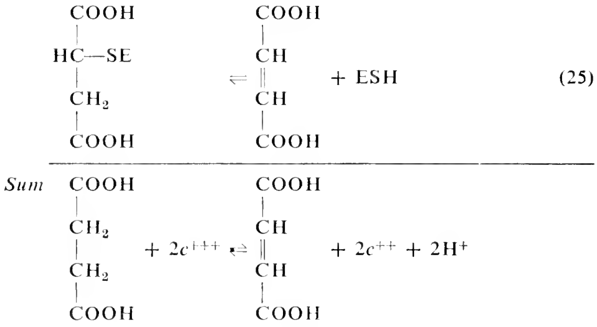
$$\mathrm{fpH_2} + b^{+++} - \mathrm{I_2}^+ \rightleftharpoons b^{+++} - \mathrm{I_2} + \mathrm{fpH} + \mathrm{H}^+ \qquad (21)$$

$$\mathrm{fpH} + c_1^{+++} \rightleftharpoons \mathrm{fp} + c_1^{++} + \mathrm{H}^+ \qquad (22)$$

$$b^{+++} - \mathrm{I_2} + \mathrm{CN}^- \rightleftharpoons b^{+++} - \mathrm{I_2} - \mathrm{CN}^- \qquad (23)$$

$$c_1^{++} + c^{+++} \rightleftharpoons c_1^{+++} + c^{++} \qquad (11)$$

$$b^{+++} - \mathrm{I_2} - \mathrm{CN}^- + c^{+++} \rightleftharpoons b^{+++} - \mathrm{I_2}^+ + \mathrm{CN}^- + c^{++} \qquad (24)$$



$$\begin{array}{c}\mathrm{COOH}\\|\\\mathrm{HC{-}SE}\\|\\\mathrm{CH_2}\\|\\\mathrm{COOH}\end{array} \rightleftharpoons \begin{array}{c}\mathrm{COOH}\\|\\\mathrm{CH}\\\|\\\mathrm{CH}\\|\\\mathrm{COOH}\end{array} + \mathrm{ESH} \qquad (25)$$

$$\textit{Sum}\quad \begin{array}{c}\mathrm{COOH}\\|\\\mathrm{CH_2}\\|\\\mathrm{CH_2}\\|\\\mathrm{COOH}\end{array} + 2c^{+++} \rightleftharpoons \begin{array}{c}\mathrm{COOH}\\|\\\mathrm{CH}\\\|\\\mathrm{CH}\\|\\\mathrm{COOH}\end{array} + 2c^{++} + 2\mathrm{H}^+$$

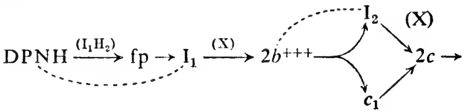
In the absence of reaction (18), the complex between fumarate and the enzyme —SH dissociates according to reaction (25).

It is, indeed, possible that this mechanism operates in the absence of cyanide and provides an alternative pathway for the oxidation of succinate not involving the reduction of the iron atom of cytochrome *b*.

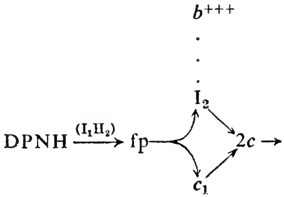
The effects of antimycin can also be adequately incorporated into these mechanisms, but that will be discussed elsewhere (Colpa-Boonstra, 1959).

The reaction mechanisms described for DPNH oxidation can be summarized (but rather inadequately) in the conventional shortened forms as follows:

Phosphorylating



Non-phosphorylating



It should be noted that the two pathways proceeding from ferrocytochrome *b* or flavoprotein to ferricytochrome *c* are not alternative, but describe two compulsorily-linked reactions proceeding at the same rate.

The chain for succinate oxidation in both phosphorylation and non-phosphorylating preparations is rather similar to the phosphorylating DPNH chain, but it cannot be adequately described in the shorthand.

Although the proposed mechanisms are certainly not the only ones which fit the observations, and the details are in many cases highly speculative, we should like to emphasize that it is our view that the true mechanisms are not less complicated than the ones proposed here.

NOTES

1. At the same time, Slater (1953) suggested that the back reaction (oxidation of reduced cytochrome *b* by fumarate) could not be ignored in any kinetic treatment of the reduction of cytochrome *b* by succinate, under these conditions. Chance (1958) has recently calculated from his new data that the back reaction was negligible in his earlier experiments. While we agree that this is a valid conclusion to be drawn from Chance's recent measurements, it is necessary to point out that Chance's calculations are based on a misreading of Slater's (1953) comment and include an arithmetical mistake of a factor of 100. He has calculated the ratio of the rates of reduction and oxidation of cytochrome *b* for the case where [succinate]/[fumarate] = 0·15, instead of [fumarate]/[succinate] = 0·15, which had been calculated by Slater from Chance's (1952) experiments. Under the conditions chosen by Chance (1958), rate of reduction/rate of oxidation = 10 × 0·15 = 1·5, not 150 given by Chance. For the conditions calculated by Slater (1953), the ratio of the rates $= \frac{10}{0{\cdot}15} = 67$, which is indeed strongly in favour of reduction.

2. The fact that the results plotted in this way fall on a straight line shows that one molecule of succinate reduces two molecules of ferricytochrome *b*, under these conditions.

3. Indeed, it seems not unlikely that the oxidation of DPNH in a normal heart-muscle preparation proceeds partly along this pathway involving reduction of cytochrome *b*, and partly by the sequence of reactions (4)–(16) shown above. We have observed that cytochrome *b* is reduced more rapidly and completely in those preparations which have the highest DPNH oxidase activity.

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DISCUSSION

The Crossover Theorem and Sites of Oxidative Phosphorylation

CHANCE: Application of the crossover theorem to the respiratory chain indicates that there are three inhibitory interactions

$$\mathrm{DPNH} \overset{\downarrow}{\rightharpoonup} \mathrm{fp} \rightharpoonup b \overset{\downarrow}{\rightharpoonup} c_1 \rightharpoonup c \overset{\downarrow}{\rightharpoonup} a \rightharpoonup a_3 - \mathrm{O}_2$$

This result immediately places considerable constraint on acceptable hypotheses for oxidative phosphorylation, since chemical equations for the relief of inhibition of electron transfer caused by adding ADP must involve interaction with these couples.

$$\mathrm{DPNH} + \mathrm{fp} \rightharpoonup \mathrm{DPN} + \mathrm{rfp} \qquad (1)$$

$$b^{++} + c_1^{+++} \rightharpoonup b^{+++} + c_1^{++} \qquad (2)$$

$$c^{++} + a^{+++} \rightharpoonup c^{+++} + a^{++} \qquad (3)$$

From Slater's paper, the couples involved are DPN and oxidized cytochrome b in his reaction (5),

$$\mathrm{DPN} \sim \mathrm{IH} + 2b^{+++} - \mathrm{I}_2 \overset{\mathrm{X}}{\rightleftharpoons} \mathrm{DPN}^+ + 2b^{++} - \mathrm{I}_2 \qquad (5)$$

and b and c, in his reaction (9),

$$b^{+++} - \mathrm{I}_2 + c^{+++} \overset{\mathrm{X}}{\rightleftharpoons} b^{+++} - \mathrm{I}_2^{+} + c^{++} \qquad (9)$$

Although the crossover data do not eliminate the possibility of such interactions, it seems only reasonable to consider first the interactions for which experimental evidence is available.

The Oxidation-reduction Potential of Cytochrome b

GEORGE: Have the various determinations of E_0' for cytochrome b all been made under the same conditions of temperature, ionic strength and pH? These factors influence the magnitude of oxidation-reduction potentials, and small differences might be partly if not wholly responsible for the discrepancies in the values.

SLATER: The main reason for the discrepancy between Ball's (1938) value of −40 mV at pH 7·4 and the mean value of +77 mV obtained by Holton and Colpa-Boonstra at pH 7·0, appears to be that Ball based his oxidation-reduction calculation on the assumption that all the cytochrome b reduced by $Na_2S_2O_4$ is functional in the succinate oxidase system. Chance has shown that this is not the case and we have confirmed this. The observations of Holton and Colpa-Boonstra showed that if no fumarate was added cytochrome b was about 98 % reduced, and not 75 % as estimated by Ball. Taking this into account raises his calculation of the potential from −40 mV to +32 mV at pH 7·4. Assuming that the potential of cytochrome *b* itself is the same at pH 7·0 as at pH 7·4 and allowing for the fact that at pH 7·0 the potential of the succinate/fumarate system is some 24 mV more positive than at pH 7·4, Ball's data correspond to a potential of +56 mV at pH 7·0. Finally it may be noted that Holton and Colpa-Boonstra's calculations are based on the data of Borsook and Schott (*J. biol. Chem.* **92,** 535, 1931) for the potential of the succinate/fumarate system (+24 mV at pH 7·0), while Ball used a value corresponding to +4 mV at pH 7·0. Thus, calculated on the same basis and allowing for the endpoint error in Ball's work, his observations correspond to a value of about +76 mV at pH 7·0, in very close agreement with the estimate of +77 mV made by Holton and Colpa-Boonstra. Chance's data also give practically the same value. Thus, there is no discrepancy between three sets of data. The difference lies in interpretation. There does appear to be some discrepancy with Hill's data, but discussion of possible reasons must await publication of details of his measurements. (See Holton and Colpa-Boonstra (*Biochem. J.* **76,** 179, 1960).)

On the Redox Potential of Cytochrome b, *the Kinetics of Reduction of Cytochrome* b, *and the Existence of Slater's Factor*

CHANCE: I am quite in sympathy with the approach of Holton and Colpa-Boonstra, to the question of how to interpret the succinate-fumarate titration of cytochrome *b* of the Keilin and Hartree heart-muscle preparation and have been actively considering this ever since we found that a dithionite-reducible pigment would cause errors in the redox potential of cytochrome *b* (Chance, *Nature, Lond.* **169,** 215, 1952). In fact the chief reason for a delay in publishing the results on cytochrome *b* titrations was that the kinetic and equilibrium data were inconsistent. I have refrained from calculating an oxidation-reduction potential, since, regardless of admonitions to the contrary, it would ultimately appear in various tables as a firmly established value. The data which have caused me to hesitate to derive the thermodynamic quantity are:

(1) The failure to obtain a reduction of cytochrome *b* by redox couples of higher potential. The 100 mV figure for the antimycin-A treated material (Slater and Colpa-Boonstra, this volume, p. 584) suggests that ascorbate would be effective.

(2) The ratio of the kinetic constants do not equal the 'equilibrium' constant. There is the possibility of a simulated equilibrium due to a fumarate reductase or similar activity. In this case, a variable cytochrome b^{+++}/b^{++} ratio could be obtained by variation of the fumarate/succinate ratio. It is probable that a fairly sophisticated reaction sequence would have to be formulated to fit exactly the experimental data (Takamiya, *J. Biochem. Tokyo* **46,** 1037, 1959).

(3) Hill's ferric-ferrous oxalate titrations presumably involve a direct reaction with cytochrome *b*, and are therefore scarcely to be ignored. The 75 mV discrepancy between his value and that which can be calculated from my data is surely beyond any measurement error. The procedure used in Hill's study has been described in detail (Hill, R. In *Modern Methods of Plant Analysis*, Ed. Paech and Tracy, **1,** 401, 1956; Bendall and Hill, *New Phytol.* **55,** 206, 1956). It would appear that any objection to Hill's results on cytochrome *b* would also apply to his results on cytochrome b_7.

A second general point is the question of whether cyanide specifically slows the reduction of cytochrome *b*. It should be noted that we have used cyanide to measure the kinetics of reduction of cytochrome *b* of liver mitochondria, and that the rate is rapid. This point can readily be tested by using azide, hydrogen sulphide, or carbon monoxide instead of cyanide: has Colpa-Boonstra tested these to determine whether the reduction of cytochrome *b* on adding succinate is faster in the absence than in the presence of cyanide?

The suggestion that cyanide is an inhibitor of cytochrome *b* reduction would allow an even more critical test of the idea of a cytochrome *b* 'by-pass': it is observed that the rate of reduction of cytochrome *c* is rapid compared with that of cytochrome *b* in cyanide-treated preparations (see Chance, this volume, p. 564). Whether or not this discrepancy is due to cyanide inhibition of cytochrome *b* reduction, it is clear that electrons can be transferred much more rapidly to cytochrome *c* than to cytochrome *b*; i.e. electron transfer to cytochrome *c* does not need to pass through cytochrome *b* in the Keilin and Hartree heart muscle preparation.*

A third point refers to the Fig. 3 of the paper by Slater and Colpa-Boonstra in which the rates of reduction of cytochromes a_3 and *b* in the aerobic-anaerobic transition are compared. I would first like to point out that they may have selected from their data an experimental condition which obscures the very phenomenon to be considered, namely a measurable time difference in the start of the reduction of cytochromes *b* and a_3. Although I feel that they have an effective instrument, it may be that its response time is inadequate to show the prior reduction of cytochrome a_3 under the experimental conditions and that the slower kinetics in the presence of malonate are more faithfully recorded. We have often observed in the presence or absence of malonate the phenomenon which Slater and Colpa-Boonstra observed in the presence of malonate.

Lastly I would be pleased to hear more details of the destruction of cytochrome *b′* by BAL. How is this related to the tentative identification of the BAL-sensitive factor as a haematin? Does not this experiment remove all data in favour of a haematin 'factor'.

SLATER: Any value for the oxidation-reduction potential for cytochrome *b* must be to some degree uncertain, so long as the pure, native cytochrome is not available. However, it seems to me that determinations of the equilibrium reached with succinate and fumarate in the absence of oxygen are useful. For one thing, Holton and Colpa-Boonstra have shown experimentally for the first time that one molecule of succinate reduces two of cytochrome *b*. The oxidation-reduction potential is one of the conventional methods of expressing the value of the equilibrium constant.

The 'failure' of ascorbic acid to reduce cytochrome *b* could be a kinetic 'failure', rather than a thermodynamic difficulty. (As a matter of fact, I have reported that a faint band of cytochrome *b* appears 30 min after the addition of ascorbate to a heart-muscle preparation in the absence of air (Slater, *Biochem. J.* **45**, 1, 1949)).

In the anaerobic steady state which is reached after exhaustion of oxygen by the succinate oxidase system, the only hydrogen donor present in a concentration appreciably above that of the catalysts in the enzymic system is succinate, while the only hydrogen acceptor present in these concentrations is fumarate. A fumarate reductase could not operate in the absence of a hydrogen donor. The most that it could do would be to change the concentrations of succinate and fumarate by bringing about the following sort of equilibrium

$$\text{succinate} + \text{E} \rightleftharpoons \text{fumarate} + \text{EH}_2$$

where E represents fumarate reductase. But since the concentrations of succinate and fumarate present are far above those of any possible enzyme present, this error would be completely negligible.

We are fully aware of Hill's measurements of the oxidation-reduction potential of cytochrome *b* which are based on titrations with both ferric/ferrous oxalate, and with fumarate/succinate. Until details of his measurements are published, it is not possible to discuss the reasons for the difference, which is perhaps less than it now appears.

There is no difference between Chance and ourselves concerning the interpretation of the slow reduction of cytochrome *b* (in comparison with that of cytochromes *c* or a_3) by succinate in the presence of cyanide. Under these conditions, it is clear that cytochrome *b* is not on the pathway of reduction of cytochrome *c* by succinate. However, we do differ concerning the interpretation of the other experiments in which the reduction of cytochrome *b* is followed during the exhaustion from the suspension of oxygen, caused by the aerobic oxidation of succinate. In our view, the course of the reduction suggests that, after a very short time lag, cytochrome *b* is as rapidly reduced as cytochrome a_3. If this interpretation is correct, it follows that cyanide must be interfering with the reduction of cytochrome *b*. One possible way in which it might do this is suggested in the paper.

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* *Added in proof*. In an experiment performed after this symposium, it has been found that cytochrome *b* of heart muscle preparation is reduced at the same rate in 1·7 mM NaCN as in 30 mM NaN_3. Thus, a specific inhibition of cytochrome *b* reduction is unlikely and the idea that cytochrome *b* is 'dislocated' in non-phosphorylating preparations appears preferable (Chance, 1958a).

Whether azide, H_2S or CO have the same effect as cyanide has not yet been tested. Antimycin clearly does not. Chance's report, which I do not think has been mentioned in his papers, that the reduction of cytochrome *b* by liver mitochondria is rapid in the presence of cyanide, is another interesting example of the fact, first found by Chance, that the kinetics of cytochrome *b* are different in phosphorylating and non-phosphorylating preparations. This is one of the points which we have attempted to explain by the speculations at the end of our paper.

Figure 3 (Slater and Colpa-Boonstra, this volume, p. 582) shows, in fact, that reduction of cytochrome *b* begins a short time (about 0·25 sec) after that of cytochrome a_3. But this is to be expected if cytochrome *b* reacts at the substrate end of the chain, and cytochrome a_3 at the oxygen end. The important point, to our mind, is that after this initial lag, the reduction of cytochrome *b* follows the same course as that of cytochrome a_3. This rapid reduction of cytochrome *b* has been found by Colpa-Boonstra both with our recorder in Amsterdam, and with that of Holton which has a faster response time. The possibility that the limiting factor in the speed of the tracing pen was the response time of the instrument was excluded after a careful examination of this point by Colpa-Boonstra.

The haem which, as measured by the intensity of the pyridine haemochrome band, disappears on treatment of a heart-muscle preparation with BAL in the presence of air is probably largely accounted for by the destruction of the myoglobin and cytochrome *b′* which are present in the preparation. This destruction cannot be related to the complete inactivation of the respiratory chain which also results from this BAL treatment, unless cytochrome *b′* is in some way involved in the chain. This appears to us rather unlikely, although not impossible, in view of Chance's finding that in the presence of antimycin, cytochrome *b′* is rapidly reduced by succinate and DPNH.

Deul (1959) has evidence that, during treatment of haemoglobin with BAL, not only is a methene bridge carbon atom attacked with formation of bile pigments, but also one or both of the vinyl side chains are attacked, possibly with the formation of a thio-ether similar to that found in cytochrome *c*. We are at present investigating the working hypothesis that I_2 in our proposed mechanism is one of the vinyl groups of cytochrome *b*, and that this group is the site of action of both BAL and antimycin.

CHANCE: The position may be summed up in the following terms: Colpa-Boonstra and Slater include cytochrome *b* in the non-phosphorylating succinate oxidase system at room temperatures, but have reasons to doubt its participation in the chain in the presence of cyanide. It is my opinion that the demonstrations at low temperatures and in the presence of inhibitors, either cyanide or malonate, provide an affirmative answer to the question as to whether electrons can be transferred to cytochrome *c* without having passed through cytochrome *b*. The question of whether cytochrome *b* can ever act fast enough to transfer electrons to cytochrome *c* appears to be answered affirmatively for phosphorylating preparations, and Colpa-Boonstra and Slater feel that they have evidence that their non-phosphorylating preparations show electron transfer function of cytochrome *b* at room temperature. It is also true that various types of electron transfer particles obtained from Green's laboratory show varied responses of cytochrome *b* (Chance, unpublished data).

It is of some importance to determine whether three conditions for cytochrome *b* activity need be postulated: (1) full participation in phosphorylating systems, (2) full participation in non-phosphorylating systems, (3) no participation in non-phosphorylating systems. It is possible that there are some vestiges of energy conservation remaining in some heart muscle preparations that make it necessary to consider mechanisms only for the first and third conditions.

SLATER: The question which has interested us for some years is whether cytochrome *b* is on the main chain when succinate or DPNH is oxidized by non-phosphorylating heart-muscle preparations, under the conditions of concentration and temperature which have usually been employed in studying the succinate oxidase and DPNH oxidase in these preparations. Our recent studies have supported our original view that cytochrome *b* is involved in succinate oxidation, and not (or to only a small degree) in DPNH oxidation.

We agree that experiments with low concentrations of DPNH, and with succinate in the presence of cyanide, have shown that in these preparations reducing equivalents can be transferred to the iron atom of cytochrome *c* without having passed through the iron atom of cytochrome *b*. We agree with Chance that this is important, especially when taken in conjunction with his discovery that phosphorylating preparations behave differently. We have attempted to account for all these findings in the reaction mechanisms which we propose.

We believe that the difference in behaviour of cytochrome *b* in non-phosphorylating preparations to DPNH and succinate (in the absence of cyanide) is significant, and is connected with the fact that, in the phosphorylating system, reduction of cytochrome *b* by DPNH is associated with phosphorylation, whereas reduction by succinate is not associated with phosphorylation.

The Influence of Cyanide on the Reactivity of Cytochrome b

ESTABROOK: Slater has introduced the possible inhibitory effect of cyanide in the interpretation of Chance's kinetic studies of cytochrome *b* reduction in heart-muscle preparations. Studies (Estabrook, *J. biol. Chem.* **227**, 1093, 1957; **230**, 735, 1958) with either a cytochrome *c* deficient preparation of liver mitochondria or with a cholate preparation from heart muscle, situations in which electron transport is interrupted because of an absence of cytochrome *c* or cytochrome oxidase, show the same type of kinetics of cytochrome *b*, relative to flavoprotein and cytochrome c_1, as reported by Chance. In these studies no cyanide was added to introduce the complication mentioned by Slater. These kinetics when analysed using an analogue computer simultaneously solving the differential equations represented for a 'straight-chain' or a 'split-chain' mechanism are compatible only with the 'split-chain' mechanism inferring a by-pass of cytochrome *b*.

SLATER: Estabrook's conclusion that cytochrome *b* is by-passed in the reduction of cytochrome *c* by DPNH is, of course, in complete agreement with my earlier observations, and with our newer observations with low concentrations of DPNH. These were also made in the absence of cyanide. His comment is therefore irrelevant to the discussion with Chance, which concerned experiments with succinate as substrate.

We were naturally aware of Estabrook's important observations, which can in fact be explained more satisfactorily by the mechanism which we propose than by simpler 'straight-chain' or 'split-chain' mechanisms. Estabrook has shown that not only is cytochrome *b* by-passed in the reaction between DPNH and cytochrome *c*, but also cytochrome c_1, since in the absence of added cytochrome *c* only flavoprotein is reduced at a rate sufficient to explain the reduction of added cytochrome *c*. This can be understood if the fpH radical produced in reaction (13) reacts more rapidly with added cytochrome *c* than with the endogenous cytochrome c_1, which seems likely.

ENERGY TRANSFER AND CONSERVATION IN THE RESPIRATORY CHAIN

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INTRODUCTION

SINCE energy transfer between the respiratory carriers affords an opportunity for conservation of a portion of this energy in intermediate forms and ultimately in adenosine triphosphate (ATP), its mechanism is of fundamental importance. It is the purpose of this paper to examine available experimental data and current theories on the nature of the respiratory chain and its relation to energy conservation. The concept of a chain-like action of cytochromes was developed early in Keilin's studies (1925, 1927) on the basis of the site of action of urethane and the isolation of cytochrome *c*, and now appears well established by both direct kinetic studies of the time sequence of oxidation-reduction reactions (Chance, 1952; Chance and Williams, 1955a) and by fragmentation of the respiratory chain (Keilin and Slater, 1953; Keilin and King, 1958; Green, 1959), although the details of the order of the cytochromes in the sequence, for example cytochromes c_1 and *c* (Keilin and Hartree, 1955), and the variable function of cytochrome *b* (Slater, 1950; Tsou, 1951; Chance, 1952, 1958a; Chance and Williams, 1955a) have required special study. Indeed, the localization of sites in the respiratory chain at which energy conservation for ATP formation occurs has led to the most widespread investigation of all, and approaches ranging from dispersion in detergents (Lehninger, 1954) or fragmentation (Green, 1959) to pH-activity relationships (Hülsmann and Slater, 1957) have been employed. However, the spectroscopic response of the carriers to the presence or absence of adenosine diphosphate (ADP) or inorganic phosphate still appears to be the only method for indicating interaction sites that is directly applicable to the intact chain (Chance and Williams, 1955b; Chance, 1959a).

SPEED OF ELECTRON TRANSFER

The Oxidation of Ferrocytochrome c

The great rapidity of interaction of the 'cryptic' haem of cytochrome *c* with other haemoproteins was recognized in the work of Altschul, Abrams and Hogness (1939) who studied the oxidation of ferrocytochrome *c* with yeast peroxidase and peroxide. In fact, this interaction has been found to be a general property of peroxidases (Chance, 1951). The velocity of this reaction is so great that the size of the active centres involved was believed to exceed that of the haematin groups, although more recent estimates suggest that an active centre of about 5 sq. Å for the reaction (see discussion on steric factors) may still satisfy the experimental data (Beetlestone, 1960).

$$c'' + \text{Peroxidase Complex II} \xrightarrow{\sim 10^8} c''' + \text{Peroxidase} \qquad (1)$$

Cytochrome Oxidase

The reaction of ferrocytochrome *c* with cytochrome oxidase also appears to be rapid. It may be studied in '*c*-depleted' cytochrome chains, but this system may not require a direct interaction of the soluble material with the oxidase because of the possible presence of small residual amounts of bound cytochrome *c* (Estabrook, 1959). Thus, most kinetic studies have been carried out with cholate-treated fractions of the respiratory chain which may contain cytochromes *a* and a_3, but no detectable amounts of the other carriers (Smith and Stotz, 1954; Yonetani, Takemori, Sekuzu and Okunuki, 1958). Although considerable difficulties arise in kinetic studies of this material due to the inhibition of the reaction by cytochrome *c* (Smith and Conrad, 1956), the initial rate studies made some time ago indicated that a second order velocity constant for the reaction could be as large as 10^7 l. × moles^{-1} × sec^{-1} (Chance, 1952).

$$(a + a_3)''' + c'' \xrightarrow{10^7} c''' + (a + a_3)'' \qquad (2)$$

Independent kinetic data suggest that it is the cytochrome *a* component with which cytochrome *c* interacts (Chance, 1955):

$$a''' + c'' \xrightarrow{10^7} c''' + a'' \qquad (3)$$

This result clearly shows that reduced cytochrome *c* can react very rapidly with the oxidase, suggesting that a special lipid form of cytochrome *c* is not necessary for the electron transfer reaction (Green, 1959).

The relevance of the result on the fragmented system depends upon estimates of the effective concentration of the cytochromes as they are built into the structure of the particle. Since spectrophotometric studies have indicated that most respiratory chains consist of approximately equi-molar amounts of the several cytochromes (Chance, 1952; 1958b), it is apparent that the great majority of the carriers can exist in an 'assembly' that consists of one each of its components (*Note* 1). Estimates of the concentration of carriers in such an assembly are based upon the packing of haemoglobin in the erythrocyte and values for the cytochromes of about 10^{-6} M are computed. At these concentrations, a velocity constant of 10^7 l. × moles^{-1} × sec^{-1} would afford electron transfer at the rate required by the oxidase activity of the particle.

MECHANISM OF ELECTRON TRANSFER IN THE PARTICLES

Thermal Collisions

The reasonable agreement between the measured reaction velocity in the 'soluble' system and that calculated for a closely-packed molecular array in the particles supports the possibility that a collision mechanism can operate by rotational or vibrational motion of the carriers about their points of attachment to the structure in which they are embedded (thermal motions would give about 10^7 collisions sec^{-1} (Chance, 1959a). Further support is provided by spectroscopic studies of the oxidation-reduction states of the respiratory carriers at liquid nitrogen temperatures; the values correspond closely to those observed at room temperature, as if the transfer processes were 'immobilized' in the solid state (Chance and Spencer, 1959). (The inhibition factor exceeds 10^7-fold.) Another result of some relevance is the considerable inhibition of electron transfer by glycerol with very little change in the steady state of the carriers (Chance and Spencer, 1959). Although alternate interpretations of these data are possible, these effects of temperature and viscosity are consistent with the collision mechanism, which also appears to be an acceptable hypothesis for energy transfer in the visual receptor as reported by Hagins and Jennings (1959), who propose brownian rotation or nutation in one or two degrees of freedom as an explanation of dichroism and bleaching kinetics in the retinal rods.

Conduction Bands

Many other theories of electron transfer are under active consideration in this laboratory and elsewhere, particularly that of the participation of electron conduction bands in the protein portion of the cytochromes (Cardew and Eley, 1959; Taylor, 1959). Two problems arise in considering this process. First, it appears that all electrons transferred from substrate to oxygen pass through the haem portion of the respiratory carriers. Although this observation still merits further experimental tests, it appears to require that all electrons transferred in conduction bands be trapped in the haem groups before transfer to the next member of the respiratory sequence. Second, the profound effect of the haem upon the transfer activity of the protein does not seem to be adequately explained by the conduction-band hypothesis. However, such an explanation is attractive because it should operate effectively with completely immobilized carriers. In this connexion, the low temperature 'steady states' of the cytochromes are of particular interest because of the probability that a number of assemblies of respiratory carriers contain adjacent oxidized and reduced forms of the cytochromes in a condition that is apparently stable for several days (Chance and Spencer, 1959). This result suggests that if conduction bands participate in electron transfer, they are not the sole mechanism of transfer.

Resonance Energy Transfer

Ample evidence for resonance energy transfer from the protein to the haem of haemoproteins (Bücher and Kaspers, 1946; 1947) is provided by the quantum requirement of unity for the splitting of myoglobin-carbon monoxide by ultra-violet light. It has also been shown that the haem of the native protein quenches the fluorescence of the free protein (Weber and Teale, 1959). In fact, the transfer of the excited state to the haem is found to be 100 times more probable than the fluorescence and 20 times more probable than other competing radiationless transfers (Weber and Teale, 1959). In view of these quantitative data, we have again considered the possibility put forward by Keilin and Hartree (1953) and more recently by Dixon and Webb (1958) that anomalies in the photochemical action spectra for the relief of carbon monoxide-inhibited respiration are caused by energy transfer from cytochromes other than the oxidase to the haem-CO compound. For example:

$$\begin{tikzcd} & & & & h\nu \arrow[dl] \arrow[d] \arrow[dr] & & & \\ \text{substrate} \arrow[r] & \text{fp} \arrow[r] & b \arrow[r] & c_1 \arrow[r] & c \arrow[r] & a \arrow[r] & a_3 \arrow[r] & \text{CO} \end{tikzcd} \qquad (4)$$

Two results suggest that the protein portions of the members of the cytochrome system do not absorb energy and transfer it along the chain to the haem-CO compound. First, as indicated by the above data, the haem of the protein absorbing the light would quench the fluorescence and thus prevent energy transfer to the next member of the chain, unless the possibility of a radiationless transfer were 20 times greater for the carriers embedded in the respiratory chain than for the haem proteins studied by Weber and Teale (1959).

The latter possibility is rendered even less likely by the second consideration, which is based on the ratio of the intensities of the Soret band and the protein band (1·85), shown in Warburg's photochemical action spectrum (1949). Such a high ratio would be very unlikely if light absorption in the protein portions of other haemoproteins were also contributing significantly to the splitting of the haem-CO compound. It should be noted that this ratio (1·85) is appropriate to haemoproteins having a rather higher number of aromatic amino acids and/or a higher molecular weight than cytochrome *c*, which has a value of 3·7 (Keilin and Slater, 1953). Currently available preparations (Okunuki, Hagihara, Sekuzu and Horio, 1958) of cytochrome oxidase show a ratio of $\sim$ 0·4 and thus are not yet pure enough to be helpful on this point.

The possibility that light is absorbed by the haem and is transferred to its protein and thence to adjacent carriers seems unlikely in view of more accurate data on the photochemical action spectrum (Castor and Chance, 1955) and recent studies of photodissociation spectra in the visible region (cf. Chance and Spencer, 1959). It is very unlikely that there exist bands in the photochemical action spectrum that correspond accurately to the α-bands of cytochromes *b* or *c*, and the actual positions of the 'anomalous' bands supports this view (Keilin and Hartree, 1953). In summary, the transfer of electronic energy between the carriers of the respiratory chain by a resonance mechanism appears unlikely, due to the peculiar property of the haem itself to act as a trap for such energy, and such a process does not contribute measurably to energy transfer in respiratory activity of the cytochrome chain. This conclusion is in agreement with the previously mentioned studies of Hagins and Jennings (1959) on retinal rods. They find no system for transferring electronic excitation over distances comparable to the dimensions of the rods.

Chlorophyll–cytochrome Interactions

The photosynthetic purple sulphur bacterium, *Chromatium*, strain D, shows high efficiency in the transfer of oxidizing equivalents from bacterial chlorophyll to cytochrome (∼2 quanta/electron (Olson, 1958)). In fact, there appears to be an earlier transfer of oxidizing equivalents to cytochrome than of reducing equivalents to pyridine nucleotide (Chance and Olson, 1960).

The effect of temperature upon the light response is small in *R. rubrum* (Chance, 1957) and *Chromatium* (Olson, 1958). Recent investigations of *Chromatium* over a wide range of temperatures, including measurements of frozen bacteria (Chance and Nishimura, 1960), show the rate of the light-induced oxidation to be the same within the accuracy of the experimental data at +28° and −22°C. An example of this type of study is afforded by Fig. 1. Here the double-beam spectrophotometer is used to record a decrease of absorbancy at 423 mμ (measured with respect to 460 mμ) caused by infra-red illumination of the suspension of bacteria. With the sample at room temperature, illumination at the point 'on' causes oxidation of 'cytochrome 423' at a rate of 0·13 μmoles of Fe $l.^{-1}$ sec^{-1} (for the assumptions involved in the extinction coefficient, cf. Olson, 1958). After a steady state has been reached, the infra-red illumination is turned off and the dark reaction causes reduction of 'cytochrome 423' at the rate of 0·01 μmoles of Fe $l.^{-1}$ sec^{-1}. The same sample of bacteria is then frozen in an ice–ethanol mixture at a temperature of −22°C; after solidification has occurred, infra-red illumination again causes the abrupt oxidation of 'cytochrome 423', this time at a rate of 0·11 μmoles of Fe $l.^{-1}$ sec^{-1}, a rate that is practically the same as that measured at room temperature. The final steady state is reached somewhat more slowly than in the room temperature experiment and in accordance with the recognized diphasicity of these reaction kinetics. On cessation of illumination, the dark reaction proceeds extremely slowly, being inhibited about 10-fold by the change of temperature. This result is of considerable significance with respect to our ideas on energy transfer processes, since the very small temperature effect suggests that a chlorophyll–cytochrome transfer does not involve a 'thermal reaction'. When the small effect of temperature upon the cytochrome–chlorophyll reaction is compared with the large effect of temperature on the cytochrome–cytochrome interactions of the respiratory chain (Chance and Spencer, 1959), the fundamental difference between the two types of energy transfer processes is underlined.

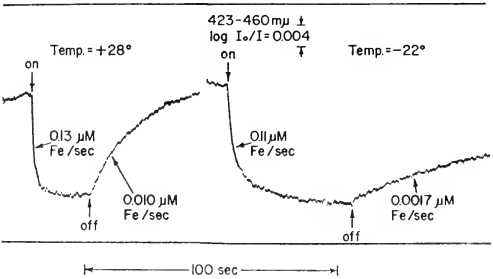

FIG. 1. A double-beam spectrophotometric recording of the effect of temperature on the light-induced oxidation of 'cytochrome 423' of the purple sulphur bacterium *Chromatium*. On the left the temperature is +28°C, and on the right the temperature is −22°C. The rates of oxidation of the cytochrome are computed on the basis of a molecular extinction coefficient of $100\ \text{cm}^{-1} \times \text{mM}^{-1}$ (Olson, 1958). The cuvette of the double-beam spectrophotometer is contained in a Dewar flask and infra-red illumination is reflected onto the sample from above (Expt. 3).

Another interesting aspect of this result is that it affords direct evidence for rapid biological processes in frozen cells as discussed by Keilin (1959).

Intercytochrome Carriers

The high velocity of the direct interaction of soluble cytochrome *c* and purified cytochrome oxidase suggests that a direct interaction of cytochromes can occur in the particles, but nevertheless intercytochrome carriers (Green, 1959) or by-passes (Martius, 1956) such as quinones have been postulated. However, titration of the inhibited respiratory chain with reduced diphosphopyridine nucleotide (DPNH) has shown that the amount of such components involved in transfers between the cytochromes (Chance, 1958b; Estabrook and Mackler, 1956) does not allow for the participation of an appreciable portion of total quinone content (Green, 1959; Redfearn, 1959). More recent studies of the sites of oxidation and reduction of ubiquinone (coenzyme Q) in the non-phosphorylating Keilin and Hartree preparation show this material to be exclusively on the substrate side of the antimycin-A sensitive point (Pumphrey and Redfearn, 1959) (cf. Equation 5). Titration studies recently conducted in this laboratory (Chance and Redfearn, in preparation), give further support to this scheme and show cytochromes *c* and *a* to be reduced completely at DPNH concentrations that cause little reduction of ubiquinone. Thus quinone does not play a part in interactions on the oxygen side of the antimycin-A sensitive point, and evidence in favour of an inter-cytochrome shuttle via a quinone system does not exist at present.

The possible function of ubiquinone remains obscure, although it could mediate flavin-cytochrome *b* interaction in phosphorylating particles. However, the situation may be different in non-phosphorylating Keilin and Hartree particles in which there is a relatively active ubiquinone reductase activity and a sluggish response of cyctochrome *b*. Nevertheless quinone function enjoys much speculation, particularly because various dehydrogenases can act as quinone reductases (Martius, 1959), for example, vitamin K_3 reductase. Slater (1959) has most recently postulated a compound of DPNH and ubiquinone in which the quinol is involved in a configuration which he terms DPN $\sim$ IH. It appears that this postulated intermediate may be similar to the inhibited form of DPNH that Chance and Williams write as DPNH $\sim$ I (see Discussion of Dickens, this volume, p. 637).

REACTION SEQUENCE OF THE CYTOCHROMES

Purification and Fragmentation

The hypothesis that purification and fragmentation of the particles does not disturb electron transfer through the components has yet to be substantiated. Indeed, contrary evidence is currently accumulating. In phosphorylating mitochondria, the kinetics of cytochrome *b* follow those of cytochrome *c* and flavoprotein with succinate or β-hydroxybutyrate as substrate (Chance and Williams, 1955a), whereas in non-phosphorylating systems such as the Keilin and Hartree heart-muscle preparation (Keilin and Hartree, 1939) cytochrome *b* clearly responds too sluggishly to participate significantly in succinate (Chance, 1952) or DPNH oxidation (Slater, 1950).

Some fragments of the respiratory chain (Green, 1959) lack cytochromes *b* or c_1, and others have relatively greater amounts of some forms of cytochrome *b* than are observed in intact beef heart mitochondria (Chance, unpublished results). For these reasons, studies of the intact electron transfer particle are highly desirable.

A second difference is the locus of the inhibitory site for Amytal in pyridine nucleotide oxidation and the comparative effectiveness of this compound as an inhibitor. Previous studies with mitochondria carrying out oxidative phosphorylation have shown (Chance, 1956) that Amytal inhibits the oxidation of DPN linked substrates at the locus of reaction of reduced pyridine nucleotide and oxidized flavoprotein, a concentration of 0·2 mM Amytal being required to cause 50% inhibition (Chance and Hollunger, in preparation). Studies on the DPNH oxidase system of a non-phosphorylating Keilin and Hartree heart-muscle preparation have shown that Amytal inhibits between flavoprotein and the cytochrome chain, with an Amytal concentration of 0·16 mM required for 50% inhibition (Estabrook, unpublished data). This modification of the locus of Amytal inhibition no doubt reflects the changes in the nature of the electron transfer chain involved in the phosphorylating and non-phosphorylating systems.

Kinetic Studies of Respiratory Components

Considerable interest is currently focused on determining both the reaction sequence of electron transfer components in the Keilin and Hartree preparation and the interaction site of ubiquinone; a closer examination of this point is now made possible by the direct spectrophotometric recording of ubiquinone reduction in collaboration with Dr. E. Redfearn. A comparison of the results of rapid chemical assays with the spectrophotometric data strongly supports the latter's applicability to the measurement of the kinetics of ubiquinone.

Summarized in Fig. 2 are four kinetic recordings representing the speed with which cytochrome *c*, flavoprotein, cytochrome *b*, and an absorption band in the ultra-violet, attributed to ubiquinone, change their intensities upon adding 4 mM succinate to a cyanide-inhibited (1·2 mM) Keilin and Hartree heart-muscle preparation. In records *A* and *C*, a downward deflection signifies an increase of absorbancy at the measuring wavelengths, 550 and 562 mμ, corresponding to the reduction of cytochrome *c* and cytochrome *b*, respectively. In records *B* and *D*, a downward deflection represents decreased absorbancy at the measuring wavelengths, 465 and 275 mμ, and thus the reduction of oxidized flavin and of oxidized ubiquinone. In view of the fact that the protein absorption interferes with the measurement of ubiquinone reduction, the reaction kinetics of this component are measured with a 63-fold dilution of the heart-muscle preparation while those of cytochrome *b*, cytochrome *c*, and flavin are measured with a 13·5-fold dilution. The results are, however, comparable if the dilution factors are taken into account. The kinetics are evaluated in terms of the initial slope of the traces which, in the case of cytochrome *c* (record A), is about as rapid as can be measured with the mixing technique employed; thus the initial slope of 2 μmoles of Fe $1.^{-1}$ $\sec^{-1}$ is an approximate value. In the case of flavin, cytochrome *b*, and ubiquinone, the initial slopes can be obtained with better accuracy. (The gaps in the traces for flavin and ubiquinone result from the stirring interval.) The results of these kinetics, all calculated to a 63-fold dilution and including additional data measured in the presence of antimycin-A, are summarized in Equation 5, where the numbers along the arrows represent the reduction rates of the component to the right of the arrow.

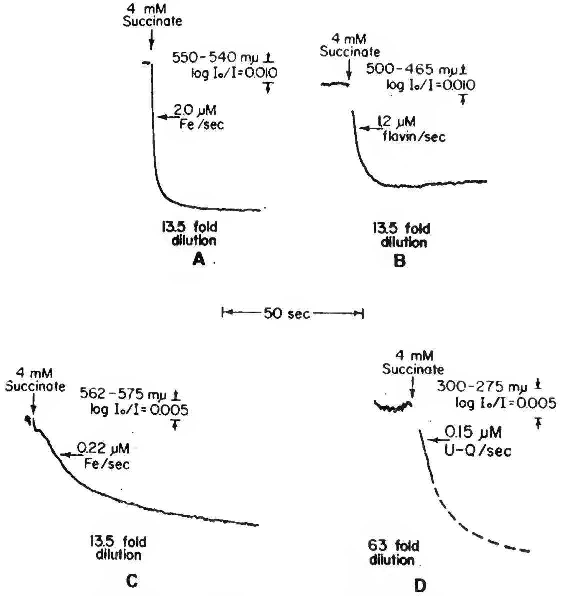

FIG. 2. The kinetics of the reduction of the cytochrome, flavin and ubiquinoue components of a Keilin and Hartree heart-muscle preparation at 12°C. In records *A*, *B* and *C* the dilution is 13·5-fold; in record *D* the dilution is increased to 63-fold. The aerobic heart-muscle preparation is pretreated with 1·2 mM cyanide and the moment of addition of 4 mM succinate is indicated by the arrow. The rates of reduction of the components, measured as soon as the mixing artifact has subsided, are given in iron atoms (1 equivalent), flavin or ubiquinone (2 equivalents) per sec. A downward deflection of the trace indicates (A) an increase of absorption at 550 mμ; (B) a decrease of absorption at 465 mμ, (C) an increase of absorption at 562 mμ; and (D) a decrease of absorption at 275 mμ. In records *A–C*, a tungsten lamp was used; in record *D* a hydrogen lamp was used. The U–Q rate was computed on the basis of $\Delta\varepsilon = 11\ \text{cm}^{-1} \times \text{mM}^{-1}$ (Expt. 989c).

All rates are given in terms of two electrons per second. Thus the rates of reduction of flavin and ubiquinone are measured directly as in Fig. 2, while those of cytochrome are divided by two.

$$\begin{tikzcd}
 & & \text{UQ} \arrow[dr] & & & & & & \\
\text{succinate} \arrow[r, "0{\cdot}26"] & \text{fp} \arrow[ur, "\begin{array}{c}0{\cdot}15-0{\cdot}25\\ \pm\text{AM}\end{array}"] \arrow[rr] \arrow[dd, "\begin{array}{l}0{\cdot}006\ -\text{AM}\\ 0{\cdot}04\ \ +\text{AM}\end{array}"'] & & \text{`f'} \arrow[r] & c_1 \arrow[r, "0{\cdot}20"] & c \arrow[r] & a \arrow[r] & a_3 \arrow[r, "0{\cdot}44"] & \text{O}_2 \\
 & & & \begin{array}{c}\text{`AM-}\\ \text{sensitive'}\\ \text{point}\end{array} \arrow[u] & & & & & \\
 & \text{b} & & & & & & &
\end{tikzcd} \tag{5}$$

It is seen that the rates at which flavoprotein and ubiquinone are reduced are comparable, whereas the rate at which cytochrome *b* is reduced in the absence of antimycin-A is about 40 times slower than that of the other two components and is, in the presence of antimycin-A approximately six times slower (Chance, 1958a). Similar data were used earlier (Chance, 1952) to demonstrate that cytochrome *b* is not a member of the electron transfer chain of non-phosphorylating preparations. Cytochrome *c* is reduced somewhat more slowly than flavoprotein for two reasons. First, electron transfer has proceeded farther down the chain, and second, cytochrome *a* is acting as a potent oxidant for cytochrome *c*. Even so, the rate of cytochrome *c* reduction is large compared to that of cytochrome *b* in the presence or absence of antimycin-A. In the uninhibited preparation oxygen is reduced at a rate of 0·44 μmoles of O_2 $l.^{-1}$ $sec.^{-1}$ in terms of two equivalents/sec. This value is about twice that measured for ubiquinone, in agreement with the data of Redfearn (1959). It is important to compare, in addition to the slopes of the reaction kinetics, the time for completion of the trace for the 275 mμ component with that of the cytochrome *c* component; the reduction of cytochrome *c* is complete by about the time the reduction of ubiquinone reaches its maximal velocity.

The Function of Cytochrome c_1 *in the Steady State*

Although detailed kinetic studies of the cytochrome components distinguishable at room temperature can be made on the intact respiratory chain as described above, the obscuring of cytochrome c_1 by cytochrome *c* has prevented our obtaining even preliminary information on the participation of cytochrome c_1 in electron transfer in the Keilin and Hartree heart-muscle preparation, or indeed in phosphorylating mitochondria. Indirect data on the fragmented system have led Keilin and Hartree (1955) to recommend the inclusion of cytochrome c_1 as a member of the respiratory chain, but its action in the steady state of respiration has never been reported.

Studies of the low temperature difference spectra of mitochondrial suspensions frozen rapidly from the aerobic steady state show remarkably little change in the percentage of oxidation and reduction of the cytochrome components such as *c*, *a* and *b* (Chance and Spencer, 1959). This observation leads to the supposition that the steady state of cytochrome c_1 would be similarly independent of freezing and that the ready delineation of this component at liquid nitrogen temperatures would lead to a reasonable estimate of its room temperature steady-state value. An experiment to this point is recorded in Fig. 3, which represents a frozen steady state of beef heart mitochondria (kindly supplied by Dr. D. E. Green) with succinate as substrate. (See Discussion, this volume, p. 458.)

It is seen that the α-band of cytochrome c_1 shows a clear reduction in the steady state: the value computed is 67%, which is very close to that of cytochrome *c*. On the basis of these data and those of a further experiment represented in Fig. 4, we feel that arguments in favour of considering cytochrome c_1 as a fully active member of the respiratory chain are somewhat

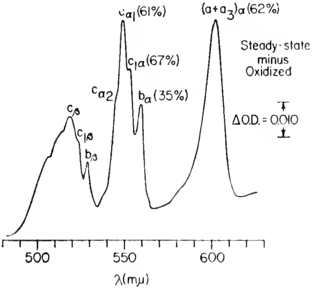

FIG. 3. A low temperature steady-state spectrum representing the difference in absorbancy between the oxidized beef heart mitochondria and the steady-state reduced beef heart mitochondria at temperatures of liquid nitrogen (77°K). The percentage reduction of the components is indicated on the figure. Succinate as substrate (Expt. 966a).

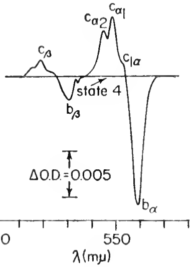

FIG. 4. A low temperature spectrum representing the difference in the steady-state oxidation level of mitochondria in state 4 and those in state 3 due to treatment with 100 μM dibromophenol. Rat liver mitochondria suspended in sucrose phosphate medium treated with 4 mM glutamate and 200 μM azide. One of the samples is treated with 100 μM DBP. Cytochrome *b* is oxidized, cytochromes *c* and c_1 are reduced (Expt. 952b).

strengthened. Ultimately, kinetic studies of this component would be highly desirable.

It is apparent that these approaches are the most suitable for providing direct evidence for the participation in electron transfer of pigments associated with the respiratory chain. Those components on which adequate data are believed to be at hand to indicate their participation in the respiratory chain of phosphorylating mitochondria of heart, liver and kidney tissues are:

$$\begin{tikzcd}
\text{succinate} \arrow[r] & \text{fp} \arrow[dr] & & & & & & \\
& & b \arrow[r] & c_1 \arrow[r] & c \arrow[r] & a \arrow[r] & a_3 \arrow[r] & \text{O}_2 \\
\text{DPNH} \arrow[r] & \text{fp} \arrow[ur] & & & & & & \\
& \text{Amytal} \arrow[u] & \text{antimycin-A} \arrow[uu] & & & & \text{CN, CO} \arrow[uu] &
\end{tikzcd} \tag{6}$$

For the intact chain, no antimycin-A sensitive factor other than cytochrome *b* need be postulated, and no postulate of an Amytal-sensitive factor between DPNH and flavoprotein has yet been made (Chance, 1956).

Since direct data do not yet show the site of action of the quinone in relation to cytochrome *b* in the phosphorylating preparations, we do not include it at present. Hatefi, Lester, Crane and Widmer (1959) maintain that coenzyme Q is located on the oxygen side of cytochrome *b*, presumably between cytochromes *b* and c_1 or *c*. This result is difficult to reconcile with previous results, with those on other preparations (Green, 1959), and with the titration data on non-phosphorylating preparations (see above).

Possible Ligands Associated with Phosphorylation

On the basis of steady-state and kinetic tests, electron transfer in phosphorylating preparations is specifically blocked in the absence of ADP or inorganic phosphate (Lardy and Wellman, 1952; Chance, 1956). Since neither ADP nor inorganic phosphate is required for electron transfer in the Keilin and Hartree preparation (Bonner, 1954) it is concluded that a substance, I, inhibits electron transfer (*Note* 2). It was further suggested by Chance and Williams (1956b) that the substance I may not be identical for the sites of energy conservation; and Slater (1958) identifies I_1, I_2, I_3, which we can label I_c, I_b and I_d to indicate the respiratory component with which the interaction occurs (Chance, 1959a). An intermediate of phosphorylation, X, formed upon addition of ADP and inorganic phosphate (P_i), reverses the inhibition caused by I by combining with it and transferring conserved energy ultimately to ADP and P_i. Electron transfer can then proceed at a rate set by the regeneration of I or X or by the rate of the intercarrier reactions (*Note* 3).

Evidence for such intermediates can be readily indicated by a comparison of the rates at which ADP and an uncoupling agent (dicoumarol) can change the steady-state oxidation-reduction level of cytochrome *c* in a preparation of phosphorylating mitochondria (Fig. 5). The aerobic suspension of mitochondria at 10°C is treated with substrate (succinate) and 250 μM ADP causes a readily measurable change of the steady state in the direction of increasing reduction at a rate of 0·1 μmoles of Fe l.^{-1} sec^{-1}. After ~100 sec, the added ADP is phosphorylated and the cytochrome is reoxidized. Addition of 22 μM dicoumarol then causes a much more abrupt reduction of cytochrome *c* (0·28 μmoles of Fe l.^{-1} sec^{-1})—almost threefold faster (*Note* 4). Similar kinetic studies have been made upon phosphate addition to the ADP-treated mitochondria.

On the basis of data of this type, equations for the interaction with the carriers can be written; (we rely upon Plaut's data on the exchange reaction (Plaut, 1957) for the order of Equations 9 and 10).

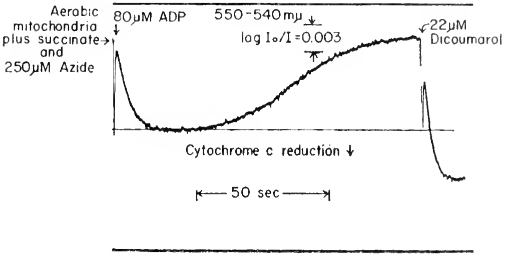

FIG. 5. A comparison of the kinetics of reduction of cytochrome *c* caused by ADP or dicoumarol addition. Succinate-treated aerobic rat liver mitochondria in salt medium (containing 0·1 $\text{M}P_i$; but fluoride-free) treated with 4 mM succinate and 250 μM azide. The additions of ADP and dicoumarol are indicated by the arrows. A downward deflection of the trace corresponds to an increase of absorbancy at 550 mμ with reference to 540 mμ. The reaction rate is measured as soon as the stirring artifact has terminated. Temperature 10°C (Expt. 525b).

$$\text{DPNH} \sim \text{I} + \text{X} \rightleftharpoons \text{DPNH} + \text{X} \sim \text{I} \qquad (7)$$

$$\text{X} \sim \text{I} + \text{DiC} \rightleftharpoons \text{X} + \text{I} + \text{DiC} \qquad (8)$$

$$\text{X} \sim \text{I} + \text{P} \rightleftharpoons \text{X} \sim \text{P} + \text{I} \qquad (9)$$

$$\text{X} \sim \text{P} + \text{ADP} \rightleftharpoons \text{ATP} + \text{X} \qquad (10)$$

Slater (1953) used the symbol C to indicate that a ligand other than phosphate interacts with the carriers. Spectroscopic studies of the steady-state oxidation levels of the carriers showed that ADP and P_i relieve an inhibition of the oxidation of reduced pyridine nucleotide in phosphorylating mitochondria (Chance and Williams, 1954), and on the basis of these data interaction of DPNH with an inhibitory ligand, I, in a configuration DPNH $\sim$ I was postulated (Chance and Williams, 1955c). Myers and Slater (1957), in an attempt to avoid multiplicity of nomenclature, used I in place of C. Lehninger, Wadkins, Cooper, Devlin and Gamble (1958) however, do not explicitly indicate inhibitory interactions but instead use the symbol X where Slater (1953) used C.

On the basis of the lack of an effect of the oxidation-reduction state of the carriers upon the ATPase reaction (Boyer, Falcone and Harrison, 1954; Cohn and Drysdale, 1955), an additional component, X, was introduced into the reaction mechanism (cf. Slater, 1958). Chance and Williams (1955c) have used the same symbol for kinetic reasons and to avoid the fault of the Lehninger mechanism (Lehninger *et al.*, 1958) which shows an uncoupling reaction on addition of phosphate. More recently, Slater (1959) has accepted the possibility of an effect of the oxidation-reduction state of the carriers upon the dinitrophenol-stimulated ATPase activity of mitochondria at pH 7·4. Although Slater states that these results weaken the basis on which he has introduced X into the reaction mechanism, we had previously pointed out that the increased binding of *both* X and I (as carrier$\sim$I and X $\sim$ I) would occur with carrier reduction and would thereby inhibit the ATPase and exchange reactions (Chance and Hollunger, 1957b; but cf. Slater, 1958, p. 250 (see *Note* 5)).

Substances such as the hypothetical inhibitory substance, I, would provide a 2-electron intermediate in cytochrome reactions as has been pointed out by Chance and Williams (1956a), i.e. cytochrome could accept one electron and I the other.

$$c^{+3}\cdot\mathrm{I} + 2e \rightharpoonup c^{+2} \sim \mathrm{I}^{-1} \tag{11}$$

This possibility has been taken up by Slater (1959) in a speculative review and indeed has some merit since it neatly avoids the problem of the 2 to 1 electron transition between flavin and cytochrome. We feel that no experimental data allow us to distinguish between this hypothesis and the dimerization hypothesis proposed by Chance and Williams (1956a).

$$2c^{+3}\cdot\mathrm{I} + 2e \rightharpoonup 2c^{+2}\cdot\mathrm{I} \tag{12}$$

The thermodynamic difficulties implicit in single electron transfer in the cytochrome chain are no longer considered formidable in view of the ready interchange of energy conserved at one site with that at another as exemplified by the succinate-linked pyridine nucleotide reduction (Chance and Hollunger, 1957a; 1960) (see below).

REVERSAL OF ELECTRON TRANSFER

The reactions of cytochromes c and $(a + a_3)$ appear to be irreversible: for example, the reduction of oxygen to water shows no measurable reversibility. Moreover, cytochromes c and $(a + a_3)$ are completely reduced in anaerobiosis. However, cytochrome b of the Keilin and Hartree preparation can be oxidized by fumarate, although kinetic studies show that this may not be an equilibrium reaction, since the titration and kinetic data are in strong disagreement (Chance, 1958a). Another reaction was observed by Slater (1950) to occur rather slowly—the oxidation of DPNH by fumarate:

$$\mathrm{H}^{+} + \text{fumarate} + \text{DPNH} \rightleftharpoons \text{succinate} + \text{DPN}^{+} \tag{13}$$

Intact mitochondria will carry out the reverse of this reaction in a process that is found to be more sensitive to antimycin-A than to cyanide, indicating that electrons pass through some of the carriers partly in the reverse of usual electron transfer. Other studies lead us to propose the following chain (Chance and Hollunger, 1960).

$$\begin{tikzcd}
 & & c & & \\
 & & c_1 \arrow[u] & & \\
\text{succinate} \arrow[r] & \text{fp}_1 \arrow[r] & b \arrow[u] \arrow[r] & \text{fp}_2 \arrow[r] & \text{DPNH} \\
 & & \text{antimycin-A} \arrow[u] & \text{Amytal} \arrow[ul] &
\end{tikzcd} \qquad (14)$$

The reaction is, however, quite sensitive to uncoupling agents and it is found that 'high-energy' intermediates are used in this reaction. It is very unlikely that ATP or other 'high energy' phosphates can be solely responsible for this reaction; ATP formed in oxidation of other substrates such as glutamate does not cause the reaction to occur and succinate is apparently essential (Chance, 1956, Chance and Hollunger, 1957a, 1960; see *Note* 6).

A direct consequence of the finding of reversal of electron transfer in the respiratory chain mediated by 'high-energy' intermediates is that energy accumulated at one site of oxidative phosphorylation can be used to drive reactions in another portion against a thermodynamically-unfavourable gradient. This result vitiates the rigorous application of thermodynamic data to the phosphorylating respiratory chain, and the detailed calculations recently made by Slater (1958) and previously by Chance and Williams (1956a) may well be restricted in situations where phosphorylation is absent. Indeed, phosphorylation efficiencies obtained from portions of the respiratory chain may be quite different from those obtained from the intact chain because energy contributions from, or donations to, other parts of the chain may be inhibited when only part of the chain is activated.

This observation may also have a considerable influence on our ability to pin-point the exact sites where 'phosphorylation' occurs in the sense that a single electron transfer reaction provides the full value of ΔF for ATP formation. Instead, we may ultimately rely entirely upon the interaction sites defined by crossover phenomena as the locus of a 'carrier-high energy intermediate' bond of sufficient strength to inhibit electron transfer in the absence of ADP and phosphate.

Localization of Interaction Sites by Fragmentation and Inhibitor Studies

In a system of the complexity of the respiratory chain, it is clear that fragmentation cannot be effective in determining the exact sites at which the inhibiting components (I) interact. For example, the use of a solution of cytochrome *c* in the measurement of phosphorylation between cytochrome *c* and oxygen (Lehninger, 1954; but cf. Slater, 1958) tells us very little about the pair of carriers between which phosphorylation occurs, since three possibilities exist:

$$c \rightharpoonup a \rightharpoonup a_3 \rightharpoonup O_2 \tag{15}$$

In a similar fashion, no fragmentation method has identified the pair of carriers involved in the additional phosphorylation obtained in the presence of succinate, where various possibilities also exist, viz.,

$$\text{succinate} \rightharpoonup \text{fp} \rightharpoonup b \rightharpoonup c_1 \rightharpoonup c \tag{16}$$

or with DPNH-linked phosphorylation:

$$\text{DPNH} \rightharpoonup \text{fp} \rightharpoonup b \rightharpoonup c_1 \rightharpoonup c \tag{17}$$

The use of inhibitors to segregate portions of the respiratory chain is also ineffective with those compounds available at present, since specific inhibitors for each portion of the chain between the carriers are unknown. Furthermore, as indicated above, thermodynamic properties of the isolated system may be very difficult to apply to the intact system, since energy conserved at one point could supplement that conserved at another point. Thus fragmentation and inhibition have limited usefulness for 'pin-pointing' interaction sites.

MATHEMATICAL PROPERTIES OF SEQUENTIAL ENZYME SYSTEMS

The complexity of the respiratory chain and its associated reactions is sufficient that kinetic studies based on the application of a simple Michaelis representation of a one-enzyme system need considerable elaboration for their effective application to the respiratory chain. For this reason, we have spent some time in this laboratory on the study of sequential enzyme systems. The mathematical studies of complex differential equations which represent the system can be solved for limited experimental conditions, such as the steady state. In addition, some useful approximations may be obtained for a transient portion of the kinetics (Higgins, 1959), and various theorems relating the properties of the mathematical system to the experimental data have been derived. Supplementing this approach is that of the analogue computer, where a somewhat simplified representation of the electron transfer system gives kinetic and steady-state solutions useful in testing theorems based on approximate mathematical solutions or theorems derived empirically from a study of the analogue computer data. Most recently complete representations of all known components and their intermediates may be obtained by the digital computer, and on this basis much more critical tests of general theorems concerning the respiratory chain may be made.

Crossover Theorem

A new approach to the localization of interaction sites is provided by the crossover theorem (Chance and Williams, 1956a; Chance, Williams, Holmes, and Higgins, 1955; Chance, Holmes, Higgins, and Connelly, 1958; Holmes, 1959) which is based on the simple observation that reducing equivalents accumulate on the substrate side of an inhibition point and diminish on the oxygen side. This is a −, + crossover for decreasing flux through the system, the − sign denoting increased reduction, and the + sign denoting increased oxidation. While the theorem appears to be trivial for a single interaction site, it becomes sufficiently complicated for three interaction sites that both Holton (1957, 1958) and Slater (1958) have questioned the generality and applicability of the theorem. Slater has discussed the case of two crossover points and concluded that they can be explained by two sites of inhibition. We should like to point out that three crossover points are needed for a direct demonstration of three sites of inhibition, and such experiments have been carried out (Chance and Williams, 1956b; Chance *et al.*, 1958). We have also been concerned with the source of energy for the ∼I compounds, particularly in the case of DPNH, and have pointed out that the portion of the oxidation-reduction cycle at which energy is conserved need not be the reduction phase alone but could also be the oxidation phase (Chance *et al.*, 1955); both, in fact, could be involved. However, crossover data clearly indicate that energy conservation and an inhibition have occurred between a pair of carriers. We have never written an equation such as Slater's Scheme C (Slater, 1958, p. 235) in which he has assigned the total energy requirement to the β-OH → DPN reaction. Also, no crossover point has yet been observed at the fp–b couple (cf. Chance *et al.*, 1955; Chance and Williams, 1956b). Our views have been expressed in the form of Fig. 7 since 1956 (Chance and Williams, 1956a, pp. 96–7, Equations 13–15), and now we can amend Equation 15 of that communication to replace c''' by c_1''' in view of Fig. 5 above.

We first derived the theorem on the basis of physical argument; it has more recently been possible to provide proofs for a variety of conditions by means of an electronic analogue computer (Chance *et al.*, 1958) and by a rigorous derivation (Holmes, 1959; see *Note* 7). The theorem is conveniently expressed as follows.

For interactions that cause decreases of flux, we can state: (1) an interaction site lies between a − to + change in the sequence from substrate to oxygen; (2) components between oxygen and the first site will always show + changes, while those between substrate and the last site will always show − changes; (3) a crossover point near the oxygen end of the chain can be shifted to the next site of interaction by a decrease of activity in the oxidase portion of the chain and vice-versa; (4) a + to − change (reversed crossover) does not identify an interaction site.

The response of a complete representation of the chemical system operating in accordance with the law of mass action to decreases of flux caused by the simultaneous ten-fold decrease of three of the velocity constants is illustrated in Table 1. It is seen that the crossover points identified by the —, + changes can be moved from the interaction site near the oxygen side of the respiratory chain to an interaction site nearer the substrate side by a decrease in the velocity constant for the reaction with oxygen (k_9), stimulating the inhibition of the respiratory chain by the addition of azide (Chance and Williams, 1956b). Not only can the crossover point be removed by one interaction site, but it can be moved by two interaction sites with a further decrease of the velocity constants. Furthermore, with appropriate conditions, the respiratory chain can show in a single experiment not one, but two of the three possible crossover points, as indicated in the bottom row of Table 1.

TABLE 1. CROSSOVER BEHAVIOUR OF ELECTRON-TRANSFER SYSTEM CAUSED BY DECREASES OF FLUX IN RESPONSE TO CHANGES IN INTERACTION AT THREE SITES

(Courtesy *Nature, Lond.*)

Enzyme concentration 10^{-6}M. Reaction velocity constants are specified in equations or in table, in units of l. × mole^{-1} × sec^{-1}. k_2, k_4 and k_6 are decreased from 0·9 to 0·09 l. × mole^{-1} × sec^{-1} to give the steady-state concentration changes shown in units of 10^{-8} molar. Values for k_5 and k_9 are given in the table.

$$\begin{tikzcd}
S \arrow[r, "k_1=0\cdot9"] & \mathrm{RPN} \arrow[r, "k_3=0\cdot9"] & b \arrow[r, "k_5"] & c \arrow[r, "k_7=0\cdot9"] & a \arrow[r, "k_8=0\cdot9"] & a_3 \arrow[r, "k_9"] & O_2 \\
 & A \arrow[u, "k_2"'] & A \arrow[u, "k_4"'] & A \arrow[u, "k_6"'] & & &
\end{tikzcd}$$

k_5	k_9	RPN	b	c	a	a_3
0·9	0·10	−26	−47	−30	+23	+44
0·9	0·07	−12	− 5	+18	+85	+49
0·9	0·04	− 4	+14	+33	+85	+25
0·2	0·5	−10	+17	−12	+12	+ 7

This demonstration of the validity of the crossover theorem, although restricted to a sequence of a limited number of components (5 chemical intermediates), has been examined over extreme ranges of velocity constants and no inconsistencies with the theorem have been found.

The response of the cytochromes of the respiratory chain to the decrease in flux caused by the expenditure of ADP is characteristic of the phosphorylating system and has shed considerable light upon the location of interaction sites. It is now apparent that, in a wide variety of isolated mitochondria and intact cells and in some tissues, the same response is observed, and that the same sites of interaction can be identified (Table 2). This observation gives a direct answer to the question of whether or not the behaviour of the cytochrome chain is similar in the living cell and in the isolated mitochondrion. Table 2 shows separate identification of three crossover points in isolated

TABLE 2. CROSSOVER BEHAVIOUR OF THE CHEMICAL SYSTEM: CHANGES OF STEADY-STATE LEVEL CAUSED BY A DECREASE OF FLUX RESULTING FROM EXHAUSTION OF ADENOSINE DIPHOSPHATE

Key: − corresponds to a reduction; + to an oxidation; 0 to no observable change.

Material	Source	Substrate	Inhibitor	Temp. °C	$S \longrightarrow RPN$	$\longrightarrow fp$	$\longrightarrow b$	$\longrightarrow c_1 + c$	$\longrightarrow a$	$\longrightarrow a_3 \longrightarrow O_2$	Reference
Mitochondria	Rat liver	β-Hydroxybutyrate	None	26	−	−	−	−	| +	+	Chance and Williams, 1955b
	Rat liver	β-Hydroxybutyrate	Azide	2	−	−	− |	+	+	+	Chance and Williams, 1956
	Rat liver	β-Hydroxybutyrate	None	26	−	| +	−	−	+		Expt. 377
	Rat liver	β-Hydroxybutyrate	None	38	−	| +	−				Chance and Baltscheff-sky, 1956
	Guinea pig liver	Succinate	None	26	−	−	−	−	| +		Expt. 376b
	Guinea pig liver	Succinate	Azide	26	−	| +	0	+	+		Expt. 384
	Guinea pig liver	Glutamate	None	26	−	−	− |	+	+	+	Expt. 372
	Guinea pig liver	Glutamate	None	26	−	| +	− |	+	+		Expt. 376b
	Guinea pig liver	Glutamate	Azide	26	−	| +	0	+	+	+	Expt. 384
	Locust flight muscle	α-Glycerol-phosphate	None	20	−	−	−	−	| +		Klingenberg, 1958
Intact cells	Yeast	Ethanol	None	26	−	−	−	−	| +		Expt. 493
	Yeast	Ethanol	Azide	26	−	| +	− |	+	+		Expt. 907
	Ehrlich ascites tumor	Glucose	None	26	−		−	−	| +		Chance and Hess, 1959
	Frog sartorius muscle	Glucose	None	26	−		−	−	| +		Jöbsis and Chance, 1957; Weber, 1957
	Frog sartorius muscle	Glucose	Azide	26			0	+	+	+	Weber, 1957
	Toad heart muscle	Glucose	None	26	−	−	− |	+	+	−	Ramirez, 1958

mitochondria by altering either the type of substrate or the extent of inhibition of the oxidase. In two cases, double crossover points have been identified under given experimental conditions in guinea pig liver mitochondria and in intact yeast cells—one between pyridine nucleotide and flavoprotein, and one between cytochromes b and $(c + c_1)$.

The crossover theorem does not specify a maximum number of interaction sites, and it is of interest to note that the data of Ramirez on toad heart muscle (Ramirez, 1959) suggest an additional site on the oxygen side of cytochrome a_3. This site, observed with considerable experimental difficulty in the intact toad heart muscle, was apparently not present in mitochondria isolated from this tissue.

It is significant that a crossover point between flavoprotein and cytochrome b has never been observed in any of the wide range of experimental conditions or materials we have studied. This point is particularly relevant to the proposal of Löw, Ernster, Lindberg, Grabe and Siekevitz (1958) that an interaction site exists between these two components. Crossover data provide no evidence in favour of this site (cf. Scheme C, Slater (1958)).

The room temperature spectroscopic data do not permit us to distinguish cytochromes c and c_1 of the phosphorylating preparation, and we have consistently lumped cytochromes c and c_1 together in our studies so far. Recent developments of the 'frozen steady state'-method now permit us to distinguish a cytochrome b–c_1 crossover point. A low temperature difference spectrum clearly shows this crossover point; the absorption band of cytochrome b is diminished and that of cytochrome c_1 is increased in material treated with ADP, relative to that without ADP. Thus the inhibitory interaction is between b and c_1. This experimental result probably requires a revision of some of the interesting speculations put forward by Slater (1959).

In summary, the experimental results on the application of the crossover theorem to intact mitochondria clearly identify three interaction sites:

(1) between pyridine nucleotide and flavoprotein;
(2) between cytochromes b and c_1; and
(3) between cytochromes c and a.

These sites have been identified in a wide variety of materials ranging from isolated mitochondria to whole tissues and under a wide variety of experimental conditions. This appears to be the only method for locating interaction sites which can be directly applied to intact mitochondria (Chance, 1959; Chance and Conrad, 1959), cells (Chance, 1959c), and tissues (Weber, 1957; Ramirez, 1959).

Representation of Reaction Kinetics

While it is of interest to propose complex reaction sequences for electron transfer and phosphorylation, they are of little use unless they can be examined in detail and compared with the experimental data. A five-component portion of the respiratory chain and three interaction sites have been represented by the analogue computer (Chance and Williams, 1956a) to demonstrate crossover phenomena, respiratory control, and phosphorylation efficiency.

The response of the steady-state oxidation levels of the components to different values of the intercarrier velocity constants is indicated in Table 1.

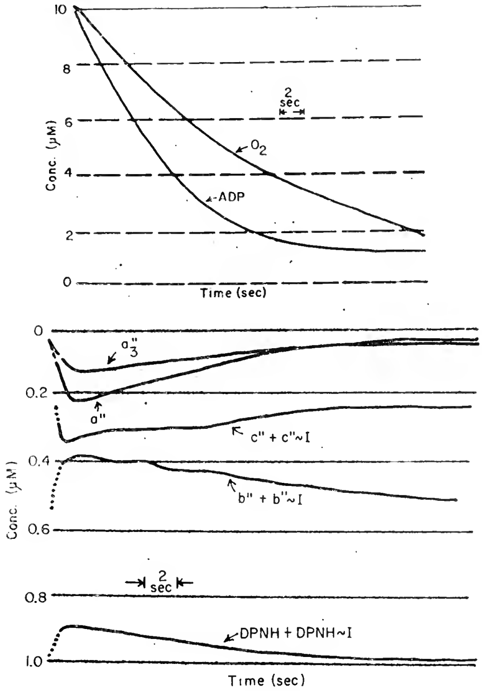

FIG. 6. An analogue computer representation of a five-membered oxidative phosphorylation chain with 3 interaction sites at cytochromes *c*, *b* and DPNH. The top traces illustrate the overall properties of the system in terms of the accelerated utilization of oxygen upon addition of ADP and the rate of utilization of ADP. The lower traces represent the changes of steady state of the respiratory carriers upon addition of ADP, a downward deflection representing an increased reduction and an upward deflection indicating an increased oxidation. It is noted that cytochrome *c* becomes more reduced and cytochrome *b* becomes more oxidized: thus a crossover point between cytochromes *b* and *c* is demonstrated. (AC–65) (Courtesy *Advances in Enzymology*)

The solution of the differential equations for the reaction kinetics of the components of a four-membered respiratory chain has been studied in some detail (Chance *et al.*, 1955) as have theorems relating the time sequence of oxidation of the components to their order in the chemical sequence. Furthermore, the time sequence of reduction of the components is clearly indicated by such studies, and theorems on the ordering of the values of $t_{\frac{1}{2}}$ 'off' have been derived in a recent dissertation (Higgins, 1959). It was on the basis of preliminary forms of such theorems that the function of cytochrome *b* in the non-phosphorylating respiratory chain could be rejected (Chance, 1952). The theorem for the ordering of the half times of the 'off' reaction can be simply stated: all intermediates (p_{ij}) involved in a linear reaction sequence have their $t_{\frac{1}{2}}$ 'off' ordered, the $t_{\frac{1}{2}}$ 'off' being greater the farther the intermediate is from the substrate. Also, for conditions which appear to be characteristic of the respiratory chain, the values of $t_{\frac{1}{2}}$ 'on' show definite ordering properties.

An example of extreme disordering in $t_{\frac{1}{2}}$ 'off' is afforded by studies of cytochrome *b* of the non-phosphorylating succinic oxidase preparation (Chance, 1952).

Not only does the analogue computer give steady-state solutions such as those in Table 1 above, but dynamic responses are also available, as illustrated in Fig. 6 where the response of the respiratory chain of Table 1 to the addition of ADP is indicated. It is seen that cytochromes $a + a_3$ and *c* become more reduced upon the addition of ADP while cytochrome *b*, pyridine nucleotide, cytochrome *c* and DPNH become more oxidized.

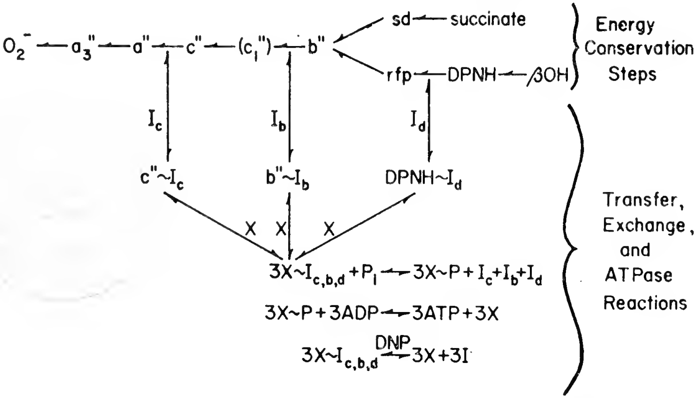
O_2^- ← a_3'' ← a'' ← c'' ← (c_1'') ← b''
sd ← succinate
rfp ← DPNH ← βOH
Energy Conservation Steps
I_c
I_b
I_d
$c''{\sim}I_c$
$b''{\sim}I_b$
$DPNH{\sim}I_d$
X
X
X
$3X{\sim}I_{c,b,d} + P_i \rightleftharpoons 3X{\sim}P + I_c + I_b + I_d$
$3X{\sim}P + 3ADP \rightleftharpoons 3ATP + 3X$
$3X{\sim}I_{c,b,d} \xrightarrow{DNP} 3X + 3I$
Transfer, Exchange, and ATPase Reactions

FIG. 7. A schematic diagram representing the electron transfer reactions, the energy conservation sites, and the phosphorylation reactions of the respiratory chain. The cytochrome components are indicated by the appropriate letter, and fp and rfp represent respectively succinic dehydrogenases and DPNH dehydrogenases. (MD–48) (Courtesy *Journal of Biological Chemistry*).

Digital Computer Solutions

Figure 7 illustrates electron transfer and phosphorylation functions of the respiratory chain. This system consists of eight respiratory components which may exist in the oxidized and reduced form, three intermediates of the respiratory carriers which may exist in 'high-energy' forms, three transfer intermediates, and a phosphorylation intermediate. The representation of the kinetics of this system by an analogue computer is not practicable, but recent work with the digital computer now makes such an approach possible (Chance, 1959c, 1960b).

NOTES

1 .The 'assembly' hypothesis has recently been termed the 'very warp and woof of the membrane fabric' (Lehninger, 1959).

2. This conclusion rests on the simplest hypothesis, namely that the non-phosphorylating preparations have a generally similar electron transfer mechanism, but that some intermediates, among them the substance I, lose their function upon preparation of the particles by fragmentation of the mitochondria.

3. It should be noted that this hypothesis is broadly based and includes that introduced by Myers and Slater (1957) in which it is assumed that electron transfer in the phosphorylating system will not proceed in the usual fashion unless a substance (which they also label I) is present. Here we are able to reconcile non-phosphorylating and phosphorylating electron transfer: electron transfer can occur without the participation of I, but no phosphorylation will result. With the participation of I, electron (or hydrogen) transfer is accompanied by energy conservation, forming a compound that inhibits further electron transfer unless reaction with the intermediate, X, occurs. This mechanism (cf. Equations 7–10) suggests that the term 'phosphorylation' should not be applied to the respiratory chain, where the energy conservation reactions do not appear to involve high-energy phosphate bonds. Furthermore, the sites at which 'phosphorylations' occur in the respiratory chain become rather meaningless and the sites of inhibitory interactions take on a greater significance than originally proposed by Chance and Williams (1955a).

4. ADP and dicoumarol concentrations are sufficient to give approximately maximal rates at 10°C.

5. There appears to be a misunderstanding of the effect of reduction of the carriers upon the rate of ^{32}P exchange or ATPase reaction. In the former case, binding of I as carrier $\sim$ I will decrease the exchange velocity in Equation 9. In addition, the increased carrier $\sim$ I concentration could easily drive more X into X $\sim$ I (as in Equation 7 above). On this basis, we have stated that under anaerobic conditions, X and I can be bound as X $\sim$ I and carrier $\sim$ I and the exchange will be slow (Chance and Hollunger, 1957a; but cf. Slater, 1958, Equation 82, p. 250 *et seq.*). In the case of the ATPase reaction, there will be no appreciable X $\sim$ I concentration due to the action of the uncoupling agent (but cf. Slater, 1958, Equations 81 and 82) and the important effect of the carriers will be expressed in their binding of I, probably in a low-energy form (carrier · I), thereby inhibiting the ATPase sequence. It seems that these data support the idea that reduced carrier binds the ligand, I, in accord with data on the ATP-jump (Schachinger, Eisenhardt and Chance, 1960) and also with spectroscopic data (Chance and Williams, 1955b; Chance, 1959c).

6. *Added in proof.* More recently it has been found that ATP can directly activate DPN reduction in succinate-treated mitochondria and that an ATP/DPNH ratio as low as 2 can be obtained (Chance, 1956; 1960a; Chance and Hagihara, 1960; Chance and Hollunger, 1960).

7. *Added in proof.* The general case for an electrical circuit consisting of *n* resistive elements and *m* interaction sites has been shown to be consistent with the crossover theorem by Dr. Hattori in this laboratory.

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DISCUSSION

Mechanism of Oxidative Phosphorylation

SLATER:

1. I do not agree with Chance that, under the conditions of the ATP-$^{32}P_i$ exchange reaction, reduction of the carrier would cause increased binding of *both* X and I (as carrier~I and X ~ I).

This exchange reaction is described, in the formulation of oxidative phosphorylation used by Chance and ourselves, by the equation

$$\text{ATP} + \text{X} + \text{I} \rightleftharpoons \text{ADP} + \text{P}_i + \text{X} \sim \text{I} \qquad (1)$$

According to Chance's mechanism, reduced carrier combines with I to form an energy-rich intermediate, (reduced carrier) ~ I. Clearly, the reaction

$$\text{reduced carrier} + \text{I} \rightarrow (\text{reduced carrier}) \sim \text{I} \qquad (2)$$

cannot occur spontaneously, but must be linked with an energy-generating or an energy-transferring reaction. In the absence of oxygen, oxidation reactions cannot provide the energy. The only possible source of this energy, in terms of Chance's mechanism, is X ~ I, which can transfer its I to reduced carrier, thus

$$\text{reduced carrier} + \text{X} \sim \text{I} \rightleftharpoons \text{X} + (\text{reduced carrier}) \sim \text{I} \qquad (3)$$

This reaction will cause no change in the amount of bound I, but will *decrease* the binding of X.

The exchange reaction (1) requires both X and X ~ I. Reaction (3) proceeding to the right will cause a decrease in the concentration of X ~ I, and an increase in that of X. Thus, on the basis of this mechanism, it is not possible to predict whether reduction of carrier would lead to an increase in the rate of the exchange reaction.

The rate-limiting step in the dinitrophenol-induced ATPase reaction is reaction (1) proceeding to the right (the back reaction can be ignored, because the concentration of X ~ I is very small). It is true, as Chance says, that binding of I by carrier in a low-energy compound (carrier · I), a reaction which could conceivably occur spontaneously, would decrease the rate of the ATPase reaction. Since, however, reduction causes a decrease of the rate of the ATPase reaction, this explanation requires that it is the reduced carrier that combines with I to form a *low-energy compound*

$$\text{reduced carrier} + \text{I} \rightleftharpoons (\text{reduced carrier}) \,.\, \text{I} \qquad (4)$$

A low-energy compound cannot itself yield ATP, but must first be converted to a high-energy compound. In oxidative phosphorylation this presumably occurs by oxidation yielding (oxidized carrier)~I. Thus, the explanation given by Chance leads to the conclusion that the high-energy intermediate I compound is with oxidized carrier, not with reduced carrier as required in his theory.

However, there are other possible explanations of the inhibition of the ATPase, for example that I is reduced. It is my view that the fact that reduction of the respiratory chain leads to an inhibition of the exchange reaction and of the ATPase provides no support for the view that ~I compounds are with reduced carrier. Nor does it prove that the compound is with oxidized carrier.

2. In my review (Slater, *Rev. pure appl. Chem.* **8**, 221, 1958) I attempted to set out as faithfully as possible Chance's mechanism of oxidative phosphorylation. Chance and Williams,(*Advanc. Enzymol.* **17**, 99, 1956) suggest that $c^{2+} \sim$ I is formed from c^{3+} and I during reduction of c^{3+} by b^{2+} and that 'similar reactions may be written for other couples'. The similar reaction leading to formation of DPN ~ I is clearly the reduction of DPN by β-hydroxybutyrate, as given in my Scheme C, which still seems to me faithfully to summarize the mechanism suggested by Chance and Williams (1956, *loc. cit.*). In the following paragraphs of my review, I discuss in detail the earlier suggestion of Chance, Williams, Higgins and Holmes (*J. biol. Chem.*

217, 439, 1955), also referred to in a footnote by Chance and Williams (1956, *loc. cit.*), that the portion of the oxidation-reduction cycle at which energy is conserved need not be the reduction phase alone, but could also be the oxidation phase. This I regard as a most useful suggestion, which we have made use of in our most recent mechanism.

3. I should prefer not to refer to C (or I) as a ligand, which I understand has a special connotation in co-ordinate chemistry. C (or I) is a group bound to a carrier.

CHANCE: The following comments concern Equations 2, 3, and 4 of the preceding remarks by Slater.

We agree most heartily that Equation 2 is irrelevant and that it is unnecessary to consider this case; we have never proposed it.

Equation 4 was introduced by us to provide an explanation for inhibition of the ATPase activity by increasing reduction of the carriers. Although this hypothesis suggests that a reduced carrier can bind I in a low-energy form, it does not exclude binding of a reduced carrier in a high-energy form and does not lead to conclusions about high-energy forms of the oxidized carrier.

The statements that Equation 3 will cause no change in the amount of bound I may be incorrect because Equation 1 clearly indicates that more I can be bound by removal of $X \sim I$ and formation of $C \sim I$. The situation is more concisely expressed by the chemical equations. (A more complete analysis is given by Boyer (*Arch. Biochem. Biophys.* **82,** 387, 1959).)

From Equation 1, the rate of utilization of ATP is

$$-\frac{d[\mathrm{ATP}]}{dt} = k_1[\mathrm{ATP}]\,[\mathrm{X}]\,[\mathrm{I}] - k_{-1}[\mathrm{ADP}]\,[\mathrm{P}]\,[\mathrm{X} \sim \mathrm{I}] \tag{a}$$

If $X \sim I$ is regulated by the reduced carrier level, c''

$$\mathrm{X} \sim \mathrm{I} + c'' \rightleftharpoons c'' \sim \mathrm{I} + \mathrm{X} \tag{b}$$

$$K = \frac{[c'' \sim \mathrm{I}]\,[\mathrm{X}]}{[c'']\,[\mathrm{X} \sim \mathrm{I}]}; \quad \mathrm{X} \sim \mathrm{I} = \frac{[c'' \sim \mathrm{I}]\,[\mathrm{X}]}{K\,[c'']} \tag{c}$$

Substituting the value of $X \sim I$ into (a), letting $k_1' = \frac{k_{-1}}{K}$, and factoring X out of both terms,

$$-\frac{d[\mathrm{ATP}]}{dt} = \mathrm{X}\left[k_1[\mathrm{ATP}]\,[\mathrm{I}] - k'_{-1}\,[\mathrm{ADP}]\,[\mathrm{P}]\,\frac{[c'' \sim \mathrm{I}]}{[c'']}\right] \tag{d}$$

The ATP^{32} exchange reaction will be regulated by whichever term of (d) is smaller. Since there appears to be an effect of the carrier concentration on the exchange reaction and since ADP and P do regulate the rate of oxidative phosphorylation and ATP does not (Chance and Williams, *Advanc. Enzymol.* **17,** 65, 1956), the second term is important for an exchange reaction that is relevant to oxidative phosphorylation.

$$\text{Exchange velocity} \propto [\mathrm{ADP}]\,[\mathrm{P}]\,[\mathrm{X}]\,\frac{[c'' \sim \mathrm{I}]}{[c'']} \tag{e}$$

Since the concentration of reduced carrier is observed to increase in anaerobiosis, a diminution of the exchange velocity is consistent with the above equations.

If reaction (b) had been written in terms of the oxidized carrier,

$$\mathrm{X} \sim \mathrm{I} + c''' \rightleftharpoons c''' \sim \mathrm{I} + \mathrm{X} \tag{f}$$

$$\text{Exchange velocity} \propto [\mathrm{ADP}]\,[\mathrm{P}]\,[\mathrm{X}]\,\frac{[c''' \sim \mathrm{I}]}{[c''']} \tag{g}$$

Since the concentration of oxidized carriers is observed to decrease in anaerobiosis, a diminution of the exchange velocity is inconsistent with the above equation (g). Thus, the "reduced carrier" mechanism is consistent with the exchange data for the conditions specified. The result does not prove that the exchange reaction follows the pathways of oxidative phosphorylation, nor does it prove that the particular mechanism is the correct one.

In view of the considerable progress made in the isolation in solution of the enzymes involved in the ATP/ADP and the ATP–P_i exchange reactions (see Plaut, *Fed. Proc.* **16,** 233, 1957 and Lehninger, *Rev. mod. Physics*, **31,** 144, 1959) it is possible to state, with certainty, that the phosphorylation of ADP and, with reasonable certainty, that the formation of 'high-energy' phosphate do not directly involve the respiratory carriers. In other words, a site of oxidative phosphorylation in the respiratory chain appears to have lost a clear meaning; we may now only attempt to identify the oxidation-reduction couple responsible for conserving the energy which ultimately allows the formation of a 'high-energy' bond. Even this identification is obscured by the conclusion based in the succinate-linked reduction of DPN: that energy conserved at one point in the respiratory chain may be used to drive energy-requiring reactions in another portion of the chain. Thus it may be very difficult to assign a particular phosphate bond to a particular site. A hypothetical example of this may be afforded by the possibility that a 'high-energy' intermediate of oxidative phosphorylation generated at a particular site which has insufficient energy to phosphorylate ADP, uses this energy to drive more reduced another component (DPN) so that a partial summation of the energies is obtained in the oxidation of DPNH. Even though this proposition has not been established experimentally, its existence as a possibility makes it much more appropriate to focus our attention upon the experimentally-determinable processes, energy conservation and inhibitory interactions (see also Chance and Hollunger, *Nature, Lond.* **185,** 666, 1960).

Inhibitory interactions and energy conservation need not occur simultaneously (see below), in fact Chance and Williams (*Advanc. Enzymol.* **17,** 65, 1955) have proposed both simultaneous and non-simultaneous mechanisms. In the absence of spectroscopically distinct forms of the respiratory carriers that indicate that energy conservation has occurred, the first available physical event discernible is that the inhibition of electron transfer has occurred.

Added in proof. The idea that the energy of a phosphate bond can be assigned to a particular half of the oxidation-reduction cycle of a carrier is unnecessarily restrictive and the so-called 'carry-over' mechanism proposed by Chance, Williams, Holmes and Higgins (*J. Biol. Chem.* **217,** 439, 1955) is probably a general mechanism of oxidative and photosynthetic phosphorylation. Designating the energy conserved in oxidation by an asterisk (*) and the total conserved following reduction by a 'squiggle' (~), we write the following equations for DPN-β-hydroxybutyrate (βOHB) interaction, the first being the energy-transfer reaction of Equation 7 above.

$$\mathrm{DPNH} \sim \mathrm{I} + \mathrm{X} \rightharpoonup \mathrm{DPNH{\cdot}I} + \mathrm{X} \sim \mathrm{I} \qquad (18)$$

the second an energy-conserving oxidation reaction:

$$\mathrm{DPNH{\cdot}I} + \mathrm{fp} \rightharpoonup \mathrm{DPN^{*}I} + \mathrm{rfp} \qquad (19)$$

and the third an energy-conserving reduction:

$$\mathrm{DPN^{*}I} + \beta\mathrm{OHB} \rightharpoonup \mathrm{DPNH} \sim \mathrm{I} + \mathrm{AcAc} \qquad (20$$

The point in the oxidation-reduction cycle at which the bond between the carrier and I is strong enough to inhibit the oxidation-reduction reaction will determine whether the reduced or the oxidized carriers accumulate in state 4. Thus, if the reduced forms accumulate, the ~ form would be involved in the rate-limiting reaction with X. If the oxidized forms accumulate, the * form would be involved in the reaction with X. It is, however, unlikely that both the * and the ~ forms would be simultaneously involved in the X reaction, since only one form would have a higher energy content than the other, and the reaction with the form of greater energy content would be the preferred one.

THE SIGNIFICANCE OF RESPIRATORY CHAIN OXIDATIONS IN RELATION TO METABOLIC PATHWAYS IN THE CELL

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THE TERM 'respiratory chain' has been interpreted with various meanings by writers on this subject. For the present purpose we can adopt the definition of Slater (1958a), in his comprehensive review of the respiratory chain in animal tissues to include all those intermediates which are actual carriers of hydrogen atoms (or electrons) in the path from substrate to oxygen.

Unfortunately there is at present much uncertainty not only concerning the sequence of these carriers, but even about which of them actually are on the respiratory chain. It will be necessary first to mention some of these difficulties. The respiratory chain itself may very likely offer alternative pathways, such as that proposed by Martius (1959), for example, who considers that in mitochondria there may be two routes linking pyridine nucleotides with cytochrome *c*, only one of which (that presumed by Martius to involve vitamin K) is accompanied by oxidative phosphorylation. In the Martius scheme, either reduced di- or tri-phosphopyridine nucleotides (DPNH or TPNH; Martius and Märki, 1957) can reduce vitamin K by means of a specific flavoprotein called vitamin K reductase (fp_6, Fig. 1), which is blocked by very low concentrations of dicoumarol (ca. 10^{-6} M), and oxidation of either TPNH or DPNH by this route should therefore produce high energy phosphate. However, the weight of the present evidence is against this stage of TPNH oxidation as a source of 'high energy' phosphate (see later). The alternative route proposed by Martius for DPNH oxidation is a non-phosphorylating one, via DPNH-cytochrome *c* reductase leading to reduction of either cytochrome *c* or c_1 (cf. Fig. 1). This reaction is thought to prevail when phosphorylation is uncoupled from respiration. A number of difficulties in the way of accepting this hypothesis, especially the lack of effect of dicoumarol on the oxidation state of cytochrome *b* (Chance) and the fact that dicoumarol blocks all three of the oxidative phosphorylation sites in the respiratory chain (Lehninger) are pointed out in the discussion of Martius's paper at the Ciba Symposium on Regulation of Cell Metabolism (1959).

Figure 1 includes a number of other possible routes of electron transport from DPNH to oxygen (the oxidation of TPNH will be mentioned separately below); oxidation of succinate via succinic dehydrogenase (fp_2) is also included. Recent work (Crane, Hatefi, Lester and Widmer, 1957; Pumphrey, Redfearn and Morton, 1958a, b; Pumphrey and Redfearn, 1959) indicates that a mitochondrial quinone (ubiquinone, Q_{275} or mitoquinone, hereafter referred to as ubiquinone) restores the activity of succinic oxidase after lipid extraction, α-tocopherol being inactive in this respect. On the other hand, α-tocopherol or vitamin K can restore the DPNH-cytochrome *c* reductase activity of aged and extracted preparations of muscle particles (Donaldson, Nason and Garrett, 1958; Deul, Slater and Veldstra, 1958). Slater (1958a) in his review of this subject discusses fully whether these quinones in fact participate in the metabolic pathways; this view is favoured by the most recent work on succinate oxidation from R. A. Morton's laboratory as far as the role of ubiquinone in oxidation of succinate is concerned (Pumphrey and Redfearn, 1959). There is also a lack of agreement about the exact site of the inhibitory action of Antimycin, which inhibits not only succinate oxidation but also that of DPNH (Slater, 1958b). The principal features of

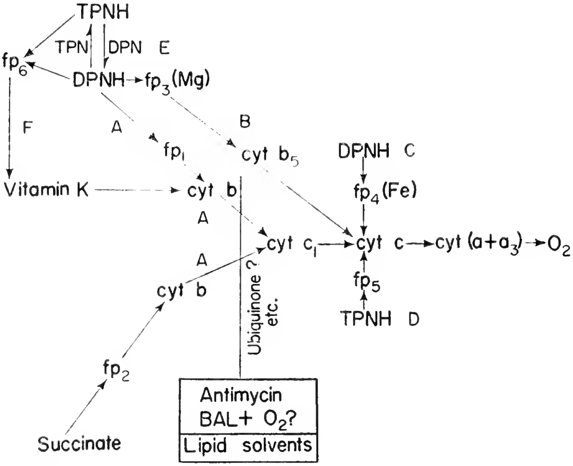

FIG. 1. Pyridine Nucleotides and Succinate in relation to the respiratory chain.
A. Mitochondria: phosphorylating: antimycin sensitive.
B. Microsomal (Strittmatter and Velick, 1956a, b; 1957a, b).
C. DPNH-cyt *c* reductase.
D. TPNH-cyt *c* reductase.
E. Pyridine nucleotide transhydrogenase (mitochondrial).
F. Vitamin K reductase (Martius).

Fig. 1 are based mainly upon Slater's 'Scheme C' (1958b, p. 187), with the introduction of cytochrome *b* into the DPNH-cytochrome *c* mitochondrial pathway in order to give a possible basis for the Antimycin sensitivity of this route.

Figure 1 shows also the recently described microsomal pathway of DPNH oxidation by means of a diaphorase (fp_3) which has been highly purified from liver microsomes by Strittmatter and Velick (1956a, b; 1957a, b) and is completely specific for the microsomal cytochrome b_5, reacting with DPNH.

The longer-known TPNH-cytochrome *c* reductases of yeast (Haas, Harrer, and Hogness, 1942) and liver (Horecker, 1950), and the iron-containing flavoprotein, DPNH-cytochrome *c* reductase, prepared from heart muscle (Mahler and Elowe, 1954) are respectively represented by fp_5 and fp_4 in Fig. 1.

A number of other flavoproteins also link with the respiratory chain, among them the acyl CoA-dehydrogenases described from D. E. Green's laboratory, which are highly important in fatty acid oxidation.

In fact, as Krebs and Kornberg (1957, p. 219) have emphasized, by means of these reactions nearly all the major metabolic sources of energy are channelled into a very few mechanisms, by transferring their hydrogen atoms (or electrons) to form reduced pyridine nucleotides or flavoproteins, which then yield up their energy, by means of the respiratory chain oxidative phosphorylations, in the form of adenosine triphosphate (ATP) required by the cell for its metabolic purposes.

There are thus three main types of compound—DPNH, TPNH and reduced flavoprotein—with which the respiratory chain has to deal. In what follows we will consider some of the factors which may affect the relative contribution of these groups to the metabolic pathways taken in animal cells.

POSSIBLE ROLES OF PYRIDINE NUCLEOTIDES IN DETERMINING METABOLIC PATHWAYS

Distribution of Pyridine Nucleotides in the Cell

Before any role of nucleotides can be discussed, it is necessary to know how much total diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) is present in cells; how much is in the oxidized and reduced forms; and what is the intracellular distribution. Until quite recently there was little such information available, but specific and sensitive methods of analysis, due to Glock and McLean (1955) in our laboratory and to Jacobson and Kaplan (1957) in Baltimore, have now partly filled this gap.

Table 1 shows some collected data for liver cells and subcellular fractions, obtained by both groups of workers. The agreement is on the whole satisfactory by these quite different methods. The values of Glock and McLean in Table 1 show several interesting points. The most obvious is the preponderance of the oxidized form of DPN over DPNH in all fractions of the liver. Quite the reverse applies to TPN which is present to a very large extent in the reduced form (TPNH) in all fractions. This implies independent operation of the two types of pyridine nucleotide systems, presumably by spatial or enzymic considerations.

TABLE 1. INTRACELLULAR DISTRIBUTION OF PYRIDINE NUCLEOTIDES IN RAT LIVER (μg/g Liver)

	DPN⁺	DPNH	TPN⁺	TPNH	mg N/g
		Glock and McLean (1956)			
Homogenate	425	116	25	231	33·6
Nuclei	22	13	5	17	7·6
Mitochondria	47	21	3	90	11·0
Microsomes	16	3	4	3	4·3
Soluble fraction	359	50	26	88	10·5
		Jacobson and Kaplan (1957)			
Homogenate	430	127	81	218	—
Mitochondria	20	6	3	67	—

After the soluble fraction, which has the highest percentage of the total DPN (75%) and of total TPN (45%), the next highest proportion of pyridine nucleotide is in the mitochondria (13% of total DPN, 36% of total TPN, mainly as TPNH). It is noteworthy that there is actually more TPN than DPN in liver mitochondria. Washing the mitochondria by suspension in isotonic sucrose containing nicotinamide and recentrifugation using the 'layering' technique, did not remove much pyridine nucleotide although some oxidation of reduced forms occurred after two such washings (Table 2 (a)). On the other hand, two similar washings of the nuclei resulted in a rather considerable fall of oxidized and reduced DPN and TPN which presumably diffuse slowly from the nuclei.

TABLE 2. (a) EFFECT OF WASHING RAT LIVER MITOCHONDRIA (0°C) ON THEIR CONTENT OF PYRIDINE NUCLEOTIDES (GLOCK AND MCLEAN, 1956) AND (b) RELEASE OF BOUND DPN AND TPN BY SHORT AEROBIC INCUBATION (30°C) WITH PHOSPHATE OR ADP (PURVIS, 1958a)

(All values in μmoles/g mitochondrial protein.)

Number of washes	DPN⁺	DPNH	(DPN⁺ + DPNH)	TPN⁺	TPNH	(TPN⁺ + TPNH)
(a) × 1	1·28	0·75	2·02	0·20	1·72	1·92
(a) × 2	1·07	0·86	1·74	0·18	1·61	1·79
(a) × 3	1·16	0·46	1·51	0·41	1·02	1·42
(b) before incubation	1·05	1·6	2·65	0·27	2·1	2·37
(b) after incubation	4·32	0	4·32	4·85	0	4·85

These results are expressed in Table 2 (a) as μmoles of pyridine nucleotide/g of mitochondrial protein (assuming a conventional protein/N ratio of 6/1). Table 2 (b) also includes measurements from Purvis (1958) on liver mitochondrial coenzymes expressed on the same basis. Apart from somewhat higher results for both DPNH and TPNH obtained by Purvis using the fluorimetric methods of Kaplan, the agreement is again satisfactory.

The last line of Table 2 (b) shows values obtained by Purvis after 5 min aerobic incubation at 30°C of the mitochondrial suspension in presence of inorganic phosphate (0·04 M), or with adenosine diphosphate (ADP, 0·002 M) in presence of nicotinamide, or with dinitrophenol. Two effects occurred: (1) the entire DPNH and TPNH became oxidized, and (2), a very remarkable result, the *total* amount of both DPN and TPN detectable in the suspension was approximately doubled. Neither the method of analysis used by Glock and McLean, nor that of Purvis, seems capable of estimating this hidden form of DPN and TPN, which Purvis suggests is the 'high energy' intermediate, (DPN $\sim$ I) or (TPN $\sim$ I), often postulated as participating in oxidative phosphorylation, and frequently discussed during this meeting. The nature of compounds causing release of free coenzyme is consistent with this idea. Direct synthesis of the extra coenzyme during incubation appears improbable since ATP, unlike ADP, is ineffective. The mechanism suggested is:

$$\text{DPNH} + \text{fp} + \text{I} \rightleftharpoons \text{DPN} \sim \text{I} + \text{fpH}_2$$

followed by release of DPN^+ from the (DPN $\sim$ I) complex by the second intermediate, X, which becomes (X $\sim$ I) and then reacts with inorganic phosphate to give (X $\sim$ P). If ADP is present as an acceptor, this complex transfers its 'high energy' phosphate to ADP forming ATP; the presence of dinitrophenol causes the breakdown by hydrolysis of the 'high energy' intermediate. In either case the bound pyridine nucleotide is set free. The estimated (DPN $\sim$ I) at 1·67 μmoles/g mitochondrial protein (Purvis; Table 2 (b)) is considered to be consistent with the content (1·5 μmoles/g protein) of 'high energy' compounds (judged by their rapid formation of ATP on addition of ADP) in similar mitochondrial suspensions, obtained previously by Eisenhardt and Schrachinger (1958). It seems to be assumed by Purvis that TPN $\sim$ I will not phosphorylate ADP. [Jacobson and Kaplan (1957, Table V) incubated liver mitochondria for 5 min at 37°C in 0·02 M phosphate, pH 7·5; they also obtained nearly complete oxidation of TPNH and DPNH but *no* increase in the total pyridine nucleotide estimated (ΔTPNH $-$ 1·25; ΔDPNH $-$ 0·12; $\Delta(\text{DPN}^+ + \text{TPN}^+)$ + 1·4 μmoles/g mitochondrial protein; calculated from these authors' data).]

These recent and important experiments have been described at some length, because if confirmed they may necessitate revision of all previous analyses of pyridine nucleotides in mitochondria. Presumably their effect on total cellular pyridine nucleotide is somewhat less serious; but even so, estimates of total cellular TPN, judging from Table 1, could be in error by as much as 100 μg/g of tissue. In tissues other than liver, the extent of bound pyridine nucleotides has not yet been determined, and this information would be very desirable.

It is just possible for example that the extremely low levels of total TPN in tumour tissue, which were consistently observed by Glock and McLean (1957), might in part be due to a high proportion of the bound form of TPN in tumour mitochondria, but this possibility has not yet been tested experimentally. Morton (1958) has already commented on the low values of DPN *per nucleus* in tumour tissue, compared with normal tissues on the same basis, and has devised an ingenious hypothesis that a low cytoplasmic DPN may in fact be a stimulus to cell division in tumours. However, when expressed on the customary basis of tissue weight, the levels of total DPN in tumours found in our laboratory was about in the middle of the range of that for normal tissues (20–27 μmoles DPN/100 g), while that of TPN was only 0·8–4·2 μmoles/100 g; nevertheless there is evidence of the existence of an active HMP shunt, which requires TPN, in tumours (Dickens, 1958). The difficulty of reconciling these facts makes the search for a hidden supply of TPN in tumours still more necessary.

Quite apart from this recent work of Purvis, it has of course long been recognized that there are differences in the accessibility of pyridine nucleotides in cells; some being bound to enzymes or cellular structure, and some in the free state. Thus Lehninger (1958) has pointed out that the phosphorylating membrane fragments, obtained by digitonin treatment of liver mitochondria, retain only one-fortieth of the DPN (relative to cytochrome *a* as standard) that the intact mitochondria possessed, yet the submitochondrial fragments have five times the activity of the whole mitochondria in oxidizing β-hydroxybutyrate. This indicates the very much higher activity of a structurally bound form of DPN, which is believed to be that concerned in oxidative phosphorylation. It also gives a further reason for the belief that only a small part of the pool of mitochondrial pyridine nucleotide is metabolically active in any given enzymic reaction. It is interesting to note that the bound malic dehydrogenase in these digitonin-treated particles did *not* react with mitochondrially bound DPN, although the bound β-hydroxybutyrate dehydrogenase readily did so (Lehninger, 1958).

As regards the oxidation of extramitochondrial pyridine nucleotides by the mitochondrial oxidative chain, this subject has been excellently reviewed recently by Ernster (1958), who distinguishes rather sharply between phosphorylating and non-phosphorylating DPNH-cytochrome *c* reductase systems. The phosphorylating type is considered to be intra-mitochondrial and to be sensitive to Amytal (believed to block the flavoprotein-DPN stage; Chance, 1956) and to Antimycin A (cf. Fig. 1). The extra-mitochondrial system requires free cytochrome *c* and oxidizes DPNH by a pathway not coupled with phosphorylation and insensitive to the above inhibitors.

Reference to Fig. 1 shows that if TPNH oxidation occurs either by the pyridine nucleotide transhydrogenase (E) or by the Martius pathway (F) its oxidation might be expected to be accompanied by a P/2e ratio of 3, just as for DPNH oxidation. As already mentioned, such ratios have not been obtained experimentally with TPNH. Table 3 shows some relevant data.

TABLE 3. OXIDATIVE PHOSPHORYLATION IN RAT MITOCHONDRIAL PREPARATIONS WITH TPNH AND DPNH

(a) VIGNAIS AND VIGNAIS (1957): SUBSTRATE ISOCITRATE

(Activity of TPNH-cytochrome *c* reductase and of DPNH-cytochrome *c* reductase was approximately equal in all 3 tissues. No exogenous coenzyme added. Note P/O ratio increases with increasing transhydrogenase activity.)

Mitochondria	Transhydrogenase activity	P/O ratio	Q_{O_2}
Brain	9	0·7	32
Liver	43	1·4	60
Kidney	129	1·8	91

(b) KAPLAN, SWARTZ, FRECH AND CIOTTI (1956): LIVER MITOCHONDRIA, DEPLETED OF COENZYMES

Addition	O_2 Uptake (μ atoms of O)	P/O ratio	α-Ketoglutarate formed (μmoles)
DPN + glutamate	1·4	2·7	0·2
TPN + isocitrate	3·2	0	2·4
TPN + DPN + isocitrate	9·6	2·3	4·3

(c) BALL AND COOPER (1957): HEART MUSCLE PARTICLES

Oxidation of TPNH occurred only after addition of DPN also; presumed therefore entirely through transhydrogenase here.

In the mitochondrial membrane fragments used by Lehninger, *added* TPNH is oxidized only if DPN is also added, and the presence of a pyridine nucleotide transhydrogenase has been directly demonstrated (Devlin and Lehninger, 1958; Devlin, 1959). Since *added* DPNH oxidation (unlike that of the internal DPNH) is not accompanied by much phosphorylation (Cooper and Lehninger, 1956) presumably 'external' DPNH and TPNH are both oxidized by the alternative non-phosphorylating route, the oxidation of TPNH proceeding via DPNH by means of the transhydrogenase reaction in this case.

The study of mitochondrial oxidation of isocitrate is important in this connexion. Both TPNH and DPNH are believed to be capable of being produced at the isocitrate dehydrogenase stage of the Krebs cycle oxidations, by two dehydrogenases each of which is specific for one of the pyridine nucleotides and both of which occur in mitochondria, while only the TPN-linked dehydrogenase is present in the soluble fraction (Ernster and Navazio, 1957). These authors found very low activities of transhydrogenase in liver mitochondria and believed, therefore, that the greater part of isocitrate oxidation proceeded via the DPN-linked isocitrate dehydrogenase. More recently Purvis (1958b) has reinvestigated this and finds a much higher transhydrogenase activity (cf. Stein, Kaplan and Ciotti, 1959), so that both in liver mitochondria and in heart sarcosomes he believes that isocitrate is oxidized almost entirely via the TPN-enzyme coupled with transhydrogenase. After depletion of pyridine nucleotides in the mitochondria by preliminary incubation at 30°C, added DPN was not reduced in presence of isocitrate until TPN was also added. The reason for the discrepancy with Ernster's findings is not clear, but may possibly find its explanation if a substance is formed from TPN (possibly TPN $\sim$ I, as already discussed) which blocks the reaction, presumably because it cannot liberate its high energy component unless DPN is also present (Purvis, 1958c). It is probable that the transhydrogenase reaction in mitochondria is much more complex than the simple reaction:

$$\text{TPNH} + \text{DPN} \rightleftharpoons \text{DPNH} + \text{TPN}$$

indicated in Fig. 1 would suggest.

A number of other possible mechanisms for interconversion of oxidized and reduced DPN and TPN have already been discussed in an earlier review (Dickens, Glock and McLean, 1959). To these should be added the system of 3-α-hydroxysteroid dehydrogenase, which acts as a transhydrogenase by reacting reversibly with both DPN and TPN, and thus resembles the previously described placental oestrone-oestradiol system, but is apparently much more widely distributed in animal tissues (Hurlock and Talalay, 1958). In the present state of ignorance of what actually constitutes the physiologically active transhydrogenase system, it is not hard to understand how such widely different results on the mechanism of TPNH oxidation could have been obtained in different laboratories, and this is likely to be an active field of future study.

REGULATION OF METABOLIC PATHWAYS THROUGH THE OXIDATION LEVEL OF TPN

One can readily see the outstanding advantage to the regulation of cellular metabolism due to the presence of the pools of the two pyridine nucleotides, DPN and TPN, of which only DPN is maintained largely in the oxidized state, is coupled directly with oxidative phosphorylation, and is oxidized by an efficient oxidation chain localized in the mitochondrial membrane. On the other hand TPNH, the predominant form of the second coenzyme, is available in the cell, even while DPN-linked oxidations are proceeding, for a series of reductive syntheses which are mainly TPNH specific. They include a number of vitally important metabolic reactions (cf. Horecker and Hiatt, 1958a, b; Dickens *et al.*, 1959):

(a) Reductive carboxylations, e.g. of pyruvate to malate—an apparently essential step in the net resynthesis of carbohydrate from pyruvate via the 'malic' enzyme, malic dehydrogenase and the reaction of Utter and Kurahashi (1954), leading from oxoloacetate to phospho*enol*pyruvate.

(b) Reductive amination of ketoglutarate to glutamate via glutamic dehydrogenase. By transaminase action on glutamate, many other amino acids are synthesized.

(c) Biological hydroxylation reactions of steroids, aromatic compounds, etc., of the general type:

$$\text{TPNH} + \text{H}^+ + \text{O}_2 + \text{phenylalanine} \rightarrow \text{TPN}^+ + \text{tyrosine} + \text{H}_2\text{O}$$

(Kaufman, 1958).

(d) Reductive synthesis of long chain fatty acids from carbohydrates via acetyl-CoA and reversal of the degradation of fatty acids, certain reductive steps of which (e.g. of crotonyl-CoA reduction to butyryl-CoA) have been found to be TPNH specific by Langdon (1957) and by Seubert, Greull and Lynen (1957).

(e) Reduction of folic acid (and derivatives) to tetrahydrofolic acid, a coenzyme of vital importance in one-carbon transfer anabolic reactions.

(f) Ring closure hydroxylation and demethylation in the biosynthesis of cholesterol and steroids, by the action of the oxidocyclase system, which preferentially utilizes TPNH and oxygen (Tchen and Bloch, 1957a, b).

In addition to a number of examples of such TPNH-linked syntheses reviewed earlier (Dickens *et al.*, 1959), striking examples of control by TPNH in intermediary liver metabolism of cholesterol, fatty acid and protein synthesis have been presented recently by Siperstein and Fagan (1958a, b) and Wilson and Siperstein (1959).

DEPENDENCE OF THE OXIDATIVE PENTOSE PHOSPHATE PATHWAY ON THE LEVEL OF OXIDIZED TPN

It is probable that the somewhat inefficient oxidation of TPNH by the cytochrome system in cells is frequently a limiting factor in the operation of the hexose monophosphate (HMP)-shunt in those tissues where adequate dehydrogenases for glucose 6-phosphate and 6-phosphogluconate are present. In such tissues, addition of substances such as oxidation-reduction dyes, capable of catalytically reoxidizing TPNH, can cause a marked alteration of metabolic pathway from Embden–Meyerhof to the HMP-shunt. On the other hand, stimulation of processes, such as the reductive synthesis of fatty acid or of glutamate, which utilize TPNH, should also produce a similar shift of metabolic pathway. Several examples of both types of control are already known.

Thus Hers (1957) showed that addition of substrates of the TPNH-linked aldose reductase present in liver specifically increased the liberation of $^{14}CO_2$ from [1–^{14}C] glucose in liver slices. By addition of ammonium salts to yeast cells respiring in presence of glucose, Holzer and Witt (1958) produced not only an increased fermentation, but also accompanying this, a marked increase in the oxidative utilization of glucose 6-phosphate simultaneously with a sharp (30 sec) four-fold increase in the level of oxidized TPN. It was concluded that the very active TPN-specific glutamic dehydrogenase in yeast utilized TPNH and ketoglutarate, in presence of the ammonium salt, to give glutamate and oxidized TPN, thus making the latter available for oxidation of glucose 6-phosphate via the HMP-shunt.

Wenner, Hackney and Moliterno (1958) showed that addition of methylene blue or a number of other artificial electron acceptors to Ehrlich ascites cells which were respiring in presence of labelled glucose had a similar effect, and increased the oxidation of C–1 of glucose by 6–30 ×, with only a slight effect on oxidation at C–6 of glucose (cf. Cahill, Hastings, Ashmore and Zottu (1958) for liver slices). More recently, McLean (1959a, b) has studied in our laboratory the effect of phenazine methosulphate, which is known to be a particularly effective substitute-carrier for enzymes which are normally flavin-linked, and also of insulin, on the oxidative metabolism of slices of rat mammary gland. The results are summarized in Table 4.

TABLE 4. MAMMARY GLAND SLICES (RAT): ACTION OF INSULIN AND PHENAZINE METHOSULPHATE ON PERCENTAGE OXIDATION OF [1–^{14}C] GLUCOSE TO $^{14}CO_2$

(McLean, 1959a, b). 500 mg tissue; 4·5 ml Krebs-bicarbonate; 20 mg glucose (0·2 μc); $O_2 + 5\% CO_2$; 38°C.

	20 Days pregnancy	10 Days lactation
Control	2%	12%
+ Insulin (1 I.U.)	3%	28%
+ Phenazine (10^{-4} M)	13%	36%

In the pregnant, non-lactating rat insulin does not increase the rate of formation of fatty acid, and has no significant effect on the conversion of [1–^{14}C] glucose to $^{14}CO_2$. In the lactating gland, however, insulin greatly increases both fatty acid synthesis and [1–^{14}C] liberation from labelled glucose (cf. Abraham, Cady and Chaikoff, 1957).

The effect of phenazine methosulphate (10^{-4} M) on slices of the lactating gland is similar to, but even more striking than, that of insulin (Table 4).

Both substances appeared to increase the amount of glucose metabolized by the gland by way of the oxidative steps of the pentose phosphate pathway and it is suggested that this occurred by an increased rate of reoxidation of TPNH, either by electron transport via the phenazine compound, or by the increased reductive lipogenesis in the case of insulin. It will be recalled that insulin has a closely similar effect in increasing the C–1 oxidative decarboxylation of glucose in adipose tissue; so marked is this effect that it has been made the basis of an extremely sensitive assay method for the determination of levels of insulin in human blood by Martin, Renold and Dagenais (1958). Recently Ball, Martin and Cooper (1959) have confirmed these results and found a graded response on the CO_2 output between 10^{-5} to 10^{-3} units of insulin per ml (normal serum averages 10^{-4} units of insulin per ml). These authors attribute the large release of extra CO_2 to the synthesis of fat from glucose, coupled with increased activity of the oxidative pentose cycle, as described above.

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DISCUSSION

On 'Additional DPN' of Incubated Mitochondria

ESTABROOK: Dickens has mentioned the recent studies of Purvis concerning the presence of a DPN $\sim$ I component in mitochondria detectable by differential chemical analysis. Recently Purvis visited our laboratory and in collaboration with Ito and Chance we were able to confirm the appearance of additional pyridine nucleotide in fractions of rat liver mitochondria treated in phosphate buffer. This additional pyridine nucleotide released on phosphate incubation is that material labelled DPN $\sim$ I by Purvis. In order to circumvent the complexity introduced by the high content of triphosphopyridine nucleotides in liver mitochondria we turned to guinea pig kidney mitochondria where the content of TPN + TPNH represents only about 5% of the total pyridine nucleotides (Bücher and Klingenberg, *Angew. Chem.* **70,** 552, 1958). With kidney mitochondria, which show excellent control of respiration and phosphorylation concomitant with respiration, the following type of results were obtained: the results are expressed as μmoles/g of mitochondrial protein.

(a) *at time zero* DPN = 1·6
DPNH = 1·3

Total = 2·9

(b) *at time 15 min after phosphate incubation*, DPN = 3·1.

The small discrepency (0·2 μmole/g) may be considered within the experimental error of the method of analysis employed. These studies seem to question the relevance of the additional pyridine nucleotide analysable with extracts from liver mitochondria as DPN $\sim$ I and the proposed correlation with the mechanism of oxidative phosphorylation.

SLATER: I am glad to hear that Estabrook has confirmed Purvis's finding of additional DPN on incubation of liver mitochondria with phosphate. Purvis did not investigate kidney mitochondria in our laboratory. Estabrook's analysis raises two questions. One is, as he points out, why do kidney mitochondria 'which show excellent control of respiration and phosphorylation concomitant with respiration' contain little if any of the additional compound, if the latter is indeed DPN $\sim$ I as suggested by Purvis? The second is why is there so much DPN in such mitochondria if the oxidation of DPNH is inhibited? Further work is required to answer these questions. In any case, I think that we should postpone further discussion of the possible function of Purvis's compound until the completion of his attempts to isolate and characterize it.

Possible Structure of Complexes of DPN or of DPNH Involved in Oxidative Phosphorylation

CHANCE: With reference to the paper by Dickens I cannot agree with the configuration DPN $\sim$ I; firstly, because of the crossover data which show that the inhibition must lie in the reaction:

$$\mathrm{DPNH} + \mathrm{fp} \rightharpoonup \mathrm{DPN} + \mathrm{rfp}$$

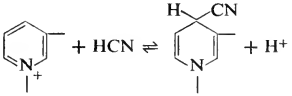
and secondly, because we do not observe inhibition of DPN reduction when substrate is added to freshly prepared mitochondria suspended in a medium free of ADP or phosphate.

Would Slater indicate the state of reduction implied by DPN $\sim$ I that is consistent with the above two considerations?

SLATER: We have written DPN $\sim$ I, because we believe that it is formed during the oxidation of DPNH to $\mathrm{DPN^+}$, thus

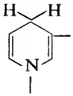
$$\mathrm{DPNH} + \mathrm{fp} + \mathrm{I\,.\,H} \rightleftharpoons \mathrm{DPN} \sim \mathrm{I} + \mathrm{fpH_2} \qquad \text{(i)}$$

DPN $\sim$ I represents a compound of oxidized DPN with I, without specifying the nature of the compound. Indeed, we always had in mind the sort of addition compound which DPN forms with HCN, thus



(ii)

which has structural and spectroscopic resemblances to DPNH



I have understood Chance's DPNH $\sim$ I to refer to a compound of reduced DPN with I, which if it were formed in a way comparable to that described in reaction (i), must arise during the reduction of $\mathrm{DPN^+}$ by substrate, for example

$$\beta\text{-hydroxybutyrate} + \mathrm{DPN^+} + \mathrm{I} \rightleftharpoons \text{acetoacetate} + \mathrm{DPNH} \sim \mathrm{I} + \mathrm{H^+} \qquad \text{(iii)}$$

We believe that this reaction is rather unlikely.

In our newer mechanism, we write I in a reduced form IH_2, so that DPN $\sim$ I becomes DPN $\sim$ IH, which is meant to represent a compound of oxidized DPN with IH_2. As before, we envisage that it is formed during the oxidation of DPNH to DPN^+.

The only difference between the new formulation and the old is that the 'I' attached to the DPN^+ is now written with an oxidizable H atom. We regard it as a compound between DPN and IH_2, rather than between DPNH and I, since we write its cleavage as

$$\mathrm{DPN} \sim \mathrm{IH} + \mathrm{H}^+ \rightarrow \mathrm{DPN}^+ + \mathrm{IH}_2 \qquad \text{(iv)}$$

If by DPNH $\sim$ I, Chance means DPNH in which one of the two hydrogen atoms on the 4-carbon atom of the pyridine ring is replaced by I, there is no difference between his views and ours. But we should prefer to regard this as a DPN compound rather than a DPNH compound.

CHANCE: Slater's Equations (i) and (ii) require a few comments, in view of the observations of Purvis that α-oxoglutarate, glutamate or succinate increases the concentration of the hypothetical DPN $\sim$ I (*Nature, Lond.* **182,** 711, 1958). Equation (i) suggests that DPN $\sim$ I is formed in an oxidation reaction, but this appears to be a serious inconsistency. The material which is observed to increase in absorption upon addition of glutamate or succinate to mitochondria absorbs maximally at 340 mμ and fluoresces maximally at 443 mμ (Chance, in *Proc. Ciba Foundation Symposium on the Regulation of Cell Metabolism*, 1959, p. 86). However, addition compounds similar to cyanide (Equation (ii)) absorb maximally at shorter wavelengths (cf. Van Eys, Stolzenbach, Sherwood, and Kaplan, *Biochim. biophys. Acta* **27,** 63, 1958). Thus, Equation (ii) appears to be inconsistent with the direct absorption data. Addition compounds with ketones contain a reduced state of DPN as they resemble charge transfer complexes (Burton, San Pietro and Kaplan, *Arch. Biochem. Biophys.* **70,** 87, 1957).

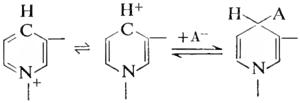
We have continuously recognized that Equation (iii) is unlikely, have never published such an equation, and have stated since 1955 (Chance, Williams, Holmes and Higgins, *J. biol. Chem.* **217,** 443, 1955): "It should be noted that the energy for the formation of the $\sim$ I need not be conserved in the oxidation-reduction reaction of Equations 8 or 10; it could have been conserved by the enzyme in a previous reaction cycle, in the chemical structure designated by the asterisk.' (See also Slater, this volume, p. 622.)

I would accept a charge transfer complex as a possible form of DPNH $\sim$ I in which the reducing equivalents resonate between the two structures:

$$\mathrm{DPNH} \sim \mathrm{I} \rightleftharpoons \mathrm{DPN} \sim \mathrm{IH}$$

provided the resonance favoured the former configuration sufficiently to agree with the physical data on absorption and fluorescence. This is, however, equivalent to saying that the physical data at present require a configuration not measurably different from DPNH $\sim$ I. It is clear that isolation of such a compound is desirable.

SLATER: I do not consider that there is any principal difference between the structure of the DPN-cyanide compound, and that of DPN-ketone compounds. The formation of both compounds may be described by the equations



where A^- is CN^- or $CH_3.CO.CH_2^-$ (for acetone), respectively. DPN-A can be regarded as an addition compound of DPN^+ and A^-, which I should prefer, or as a DPNH compound in which the H is replaced by A. It cannot be regarded as an addition compound of DPNH with A. All compounds of the type DPN-A have an absorption band in the near ultra-violet region. This is at 340 mμ when A is H— or $CH_3.CO.CH_2$—, at 330 mμ when A is RS—, and at 325 mμ when A is CN^-. Thus DPNH and the DPN-acetone compound cannot be distinguished spectrophotometrically and in fact Warburg mistakenly identified the similar DPN-glyceraldehyde compound with DPNH. Thus it is possible that DPNH and DPN ~ I might not be distinguishable spectrophotometrically. There is, therefore, no inconsistency between Chance's data and our mechanism. All compounds of the type DPN-A would also be expected to have similar fluorescence spectra. Increased DPN ~ I formation would be expected to result from the increased DPNH concentration resulting from addition of α-oxoglutarate or glutamate. The formation of DPN ~ I by addition of succinate has already been discussed by Slater (1958).

In summary, the suggestion of a DPN compound would appear to be consistent with the spectrophotometric and fluorometric data, and to conform with the known chemical properties of DPN.

Even if A contains an oxidizable hydrogen atom, as in our suggested DPN ~ IH, I cannot see a possibility of a charge-transfer complex in which the reducing equivalents resonate between the two structures:

$$\text{DPNH} \sim \text{I} \rightleftharpoons \text{DPN} \sim \text{IH}$$

since the pyridine ring in DPN-A compounds cannot easily accept an additional hydrogen atom. Thus, it appears that by DPNH ~ I Chance must mean something different from what we mean by DPN ~ IH.

CHANCE: Slater is correct in concluding that he and I have different meanings for the configurations of DPNH ~ I and DPN ~ IH; our mechanism does not require an "I" group bound in the 4-carbon atom of DPN in order to give DPN ~ IH the spectroscopic properties of DPNH. We wish to include the possibility specifically ruled out by Slater, namely an addition compound of DPNH with A or I which could be a protein group in view of the enhanced and shifted fluorescence of mitochondrial DPNH.

Slater's suggestions that DPNH and DPN ~ I might not be distinguishable spectrophotometrically and that DPN ~ I conforms with the known properties of DPN focus particular attention upon experiments in which the increase of absorption at 340 mμ caused by addition of substrates to mitochondria is compared with the diminution of absorption at the same wavelength caused by addition of acetyldehyde and alcohol dehydrogenase to an extract of the mitochondria. Such experiments have been carried out in the two laboratories where they can be appropriately made (see M. Klingenberg, W. Slenczka, and E. Ritt, *Biochem. Z.* **332**, 47, 1959 and B. Chance and G. Hollunger, *Nature, Lond.* **185**, 666, 1960).

CONCLUDING REMARKS

LEMBERG: We now come to the end of this week of hard work. My first task is to thank all the contributors who have really made this meeting a success, and the Chairmen of the Sessions who often had quite a hard task to keep us in order. I thanked the organizations which made this meeting possible at the beginning but I have still to thank my colleagues on the Organizing Committee, particularly Prof. Morton and Dr. Falk for the tremendous amount of work they have done which was—as it usually is—far greater than that done by the President. I also thank Prof. Ennor who has been very helpful to us in his capacity as Convener of the National Committee of Biochemistry. Finally, I wish to repeat the thanks which, in the name of the Organizing Committee, I have already conveyed by letters to the Staff of the Australian Academy of Science and of the C.S.I.R.O. for their very valuable help.

We have moved through a large orbit and varying orbitals, from quantum mechanics to porphyrin and protein chemistry and through biochemistry proper into the realm of biology. Evidently my appeal for brotherly love has been followed and this has contributed to the success of the meeting. Thank you all; we wish you a good and happy journey home or to any other country to which you may go from here. We hope that you have enjoyed your stay in Australia, and that you will come back to us occasionally.

GEORGE: As one of the overseas visitors I wish to express formally our thanks to the Australian Academy of Science for looking after us in such a wonderful fashion.

LEMBERG: I shall have pleasure in conveying this message to the Academy. I now declare the Haematin Enzyme Symposium of the International Union of Biochemistry closed.

SUMMARY H(A)EMATIN ENZYME SYMPOSIUM CANBERRA 1959

Have you learned your a, b, c
Cytochromes? And whether b_3
Is b_5 or inversely?
Is b_4 a 'b' or 'c'?
Is it heme, h-e-m-e
Or is it hem?
Haemo-, haemi-, ferri-
Chrome, or simply
Haemochromogen?
DPN or TPN?
Is it σ, is it π?
Is your spin low or is it high?
What the [—— ——] is I?
DNP and free-energy,
ADP and ATP
APS and PAPS
Perhaps are traps.
$a + a_3$ equals a
Follows a_3 equals 0.
Is there copper in the way?
E_0' in cell, oh no!
γ/α ratio
Crypto, cytodeutero
Spectres,
Hosts of ghosts!
$\Delta m\mu$ and Q_{O_2}
If only we knew!

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