CCXXXII. THE MECHANISM OF THE REACTION OF SUBSTRATES WITH MOLECULAR OXYGEN. I.

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Cytochrome. The most outstanding contribution to our knowledge of the mechanism of the reaction *in vivo* of substrates with molecular oxygen was the theory advanced by Keilin [1925; 1929; 1930] in which he maintained that oxidised cytochrome was reduced by substrates in presence of their respective dehydrogenases, and that reduced cytochrome was oxidised by molecular oxygen in presence of the indophenol oxidase. The reaction between substrate and oxygen was thus regarded as a process in two stages, one of which concerns the reduction of cytochrome and the other the oxidation of cytochrome. The experimental basis for this theory is briefly the following:

1. Bands of one or more of the cytochromes (*a*, *b*, *c*) are visible in practically all aerobic organisms and cells.

2. The alternate oxidation and reduction of cytochrome can be easily observed in living untreated cells of various organisms. For example by varying either the temperature or the oxygen tension of a yeast suspension, cytochrome can be converted at will from the reduced to the oxidised form or *vice versa*.

3. All factors which inhibit the activity of the dehydrogenase systems of the cell also delay the reduction of oxidised cytochrome.

4. The cells of yeast, muscle and other tissues contain an indophenol oxidase which catalyses the oxidation of both *p*-phenylenediamine and of cytochromes *a* and *c*. The activity of this oxidase is inhibited by very small concentrations of KCN and H_2S, and by CO at high partial pressure and in the dark. The oxidation of cytochrome is inhibited or abolished by the same factors which inhibit or abolish the activity of the indophenol oxidase.

5. The oxidase-cytochrome system can be reconstructed from the oxidase of heart muscle preparations, and from cytochrome *c* extracted from baker's yeast. Neither oxidase nor cytochrome alone can oxidise cysteine to any appreciable extent. When however cytochrome and oxidase are brought together, they form a powerful catalytic system which oxidises cysteine to cystine very rapidly indeed.

6. There is a marked parallelism in nature between the distribution of cytochrome, the distribution of the oxidase and the respiratory activity of the cell.

Recently Haas [1934], by the use of an elegant spectroscopic technique, attempted to measure the quantitative rôle of cytochrome in the respiration of baker's yeast. By determining the concentration of cytochrome in the yeast suspension, and the number of times per unit interval that each molecule of cytochrome is reduced and oxidised, he was able to calculate what percentage of the total respiration proceeds through cytochrome. Calculation showed that for baker's yeast at 0°, the observed rate of reduction and oxidation of cytochrome can account for the entire respiration—within the limits of experimental error.

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Yellow pigment. Warburg and Christian [1932, 1, 2] isolated a yellow pigment from bottom yeast with the following characteristics. It is a protein combined with a yellow prosthetic group and is capable of reversible oxidation and reduction. When a dilute solution of yellow pigment is added to the hexose-monophosphate dehydrogenase system of yeast or mammalian red blood corpuscles, a vigorous oxygen uptake ensues, whereas in the absence of added carrier, the dehydrogenase system is incapable of reacting with molecular oxygen. Thus the yellow pigment can function as an intermediary between hexosemonophosphate and oxygen in exactly the same fashion as methylene blue. That is to say the oxidised form of the pigment is reducible by the dehydrogenase system whilst the leuco-form is autoxidisable.

The researches of György *et al.* [1934], Euler and Adler [1934], Kuhn *et al.* [1934] and Euler, Adler and Schlötzer [1934] indicate that the yellow pigment or some simple derivative thereof is to be found in practically all types of animal, plant and bacterial cells. Warburg and Christian [1933, 2] determined the concentrations of yellow pigment in various bacteria and yeast. They found the yellow pigment in highest concentration in anaerobic organisms, and they concluded that the yellow pigment is concerned primarily in anaerobic processes. This conclusion was strengthened by the evidence of the "Wechselzahl" of the yellow pigment *in vivo*. Calculation showed that for the amount of yellow pigment present, a "Wechselzahl" of 4800 per minute was necessary in the case of baker's yeast, and of 19,000 per minute in the case of *B. pasteurianum* in order that all the respiration should proceed by way of the yellow pigment. Since the observed "Wechselzahl" of the yellow pigment when reduced by the hexose-monophosphate system *in vitro* is about 30, only 1/160th part of the respiration of baker's yeast, and 1/630th part of the respiration of *B. pasteurianum* can be assumed to involve the yellow pigment. That is to say the yellow pigment cannot be of any quantitative importance in these two highly respiring micro-organisms. But calculation showed that the respiration of *B. delbruckii* (normally anaerobic) in presence of hexosemonophosphate can be accounted for entirely on the basis of the known rate of oxidation and reduction of the yellow pigment present in the cells.

Flavin. Following Warburg and Christian's discovery of the yellow pigment Kuhn *et al.* [1933] succeeded in crystallising lactoflavin from milk and ovoflavin from dried egg albumin. Both compounds were found to have the composition $C_{17}H_{20}O_6N_4$ and to be related to the yellow pigment in much the same way as haematin to haemoglobin. Wagner-Jauregg *et al.* [1933; 1934, 1, 2, 3; 1935] investigated the power of animal cells to reduce flavin and found that tissue pulp or extracts can reduce flavin anaerobically in the presence of the following substrates—lactate, pyruvate, succinate, hexosemonophosphate, propaldehyde, glyceraldehydephosphate and citrate. However, the reduction process was rather slow compared with that of methylene blue. Analysis of the difference in the speeds of reduction of flavin by rat liver and frog muscle, both in presence of succinate, led Wagner-Jauregg *et al.* [1934, 1] to the discovery that the yellow pigment plays an important rôle in the reduction of flavin. There is apparently a deficiency of yellow pigment in frog muscle and a comparative abundance in rat liver. The ability of the yellow pigment to catalyse the reduction of flavin was well illustrated in experiments on the hexosemonophosphate system of yeast [Euler and Adler, 1934]. With all components of the system present, but without yellow pigment, the time for complete reduction of a given amount of flavin was greater than 200 min. With yellow pigment present, the same amount of flavin was reduced in 9 min. Wagner-Jauregg *et al.* further

found that the yellow pigment effect was most pronounced with the following substrates—hexosemonophosphate, hexosediphosphate, malate, lactate and citrate.

Wagner-Jauregg *et al.* [1934, 1, 2, 3] have proposed the theory "daß das gelbe Ferment[1] ein integrierende Bestandteil eine Reihe von Dehydrierungssystemen ist". It is difficult to understand the precise implication of this theory. We may conceive of the yellow pigment as actually combined with the dehydrogenase, and collaborating so to speak with the enzyme in activating substrates and catalysing the transfer of hydrogen, or we may imagine that the yellow pigment is only another link between the substrate and the oxidising agent, though in no wise associated with the enzyme. That is to say the dehydrogenase system reduces the yellow pigment, and the leuco-pigment formed reduces methylene blue, flavin or oxygen directly. Wagner-Jauregg further suggests that the cyanide-stable portion of respiration probably involves yellow pigment and flavin catalysis.

Adler and Euler [1934; 1935] have demonstrated that the enzymic oxidation of both alcohol and glucose either by methylene blue, lactoflavin or molecular oxygen is catalysed by the yellow pigment. These investigators assume as well that the yellow pigment forms some sort of combination with the dehydrogenase and is in the strict sense of the word a true enzyme.

Glutathione. Hopkins [1929] isolated crystalline glutathione from yeast, and Meldrum and Dixon [1930] studied its catalytic properties. The latter found that the autoxidation of glutathione depends on the cooperation of two factors, present in traces as impurities in the glutathione preparations: namely iron (or copper) and some substance able to form catalytically active complexes with metals. The rate of oxidation of crystalline glutathione is limited by the amount of the second factor present, and not by the iron. "Thermostable tissue preparations" can reduce oxidised glutathione although the tissue preparation inhibits the autoxidation of reduced glutathione. Certain other proteins behave like the thermostable preparations in inactivating glutathione, as does fresh washed muscle. Hopkins and Elliot [1931], in a study of the relation of glutathione to cell respiration, established the following. (1) The reduction of disulphide in mammalian liver proceeds with great velocity and is so much more rapid than the oxidation process that all the glutathione remains as —SH until the store of metabolites is almost entirely depleted. (2) The reduction process is enzymic and is presumably brought about by certain of the dehydrogenase systems. The oxidation of reduced glutathione is non-enzymic as shown by the fact that the rate of oxidation remains unchanged after the tissue has been heated to 70° for an hour. The oxidising system is sensitive to cyanide and is probably iron in combination with cysteine.

Mann [1932] found that glutathione is reduced by glucose in presence of the glucose dehydrogenase, and more recently Meldrum and Tarr [1935] found that

[1] The introduction into the literature of terms such as yellow enzyme and "Zwischenferment" has brought about a great deal of confusion. The reference to the yellow pigment as an enzyme is purely on the basis of its protein nature and heat-lability. To be consistent, cytochromes *a* and *b* should likewise be called enzymes. It would be far more satisfactory and less confusing to reserve the term enzyme in the case of oxidation systems for the material which activates the substrate, and to refer to substances such as yellow pigment, cytochrome *etc.*, merely as carriers. It is also regrettable that the new term "Zwischenferment" should be used to describe a dehydrogenase. Inasmuch as the term dehydrogenase has been used extensively in the last decade and has the virtue of specifying the nature of the catalysis, there is little reason for any change in nomenclature.

the hexosemonophosphate system of yeast or mammalian red blood corpuscles can reduce glutathione very rapidly indeed.

There are thus four generally distributed carriers which are presumed to be concerned in cellular respiration and it was the purpose of our experiments to determine to what extent these carriers can account for the reaction of substrates with molecular oxygen. In our opinion, the evidence for the rôle of any of these carriers was rather fragmentary and required considerable amplification.

It is noteworthy that Keilin has shown that the reduction of cytochrome *in vivo* takes place in the presence of lactate, succinate and glucose only. There are no data as to whether all the dehydrogenase systems as a class can reduce cytochrome or can utilise the cytochrome-indophenol oxidase system for the reaction with molecular oxygen. Harrison [1931] reconstructed the system glucose oxidase-cytochrome-indophenol oxidase and claimed to have found it very active catalytically, but our experiments indicate that this effect may not be real.

The theory that the yellow pigment is an integral part of the dehydrogenase complex rests entirely on the experimental fact that methylene blue or lactoflavin is reduced more rapidly by dehydrogenase systems in presence of the yellow pigment than in its absence. This fact might equally well be interpreted to show that the yellow pigment is reduced more rapidly than either methylene blue or lactoflavin, and that the leuco-pigment in consequence of its negative potential can reduce directly either of the hydrogen acceptors. Considered from this point of view, the yellow pigment functions purely as a carrier and not as an enzyme. What is perhaps the weakest point of the theories of Wagner-Jauregg and Euler is that although *in vitro* yellow pigment can catalyse the cyanide-insensitive oxidation of both hexosemonophosphate and alcohol, *in vivo* both processes are completely arrested by small concentrations of cyanide. The possibility must therefore be considered that the observed effects of yellow pigment may be artificial in the sense that the catalysis does not take place within the cell.

In the first part of this paper, the effects of glutathione, flavin, yellow pigment and cytochrome on the oxygen uptake of most of the known dehydrogenase systems are described. The second part includes miscellaneous experiments dealing with the general question of the mechanism of the utilisation of molecular oxygen by dehydrogenase systems.

Experimental.

Manometric measurement of oxygen uptake. The usual Barcroft technique was employed with KOH-soaked filter paper in the centre pot for the absorption of CO_2 [Dixon, 1934]. The experiments were performed at 37°. The substrate was usually pipetted into Keilin cups and introduced into the main body of fluid after equilibration. All experiments were always performed at least twice and in most cases more often. With oxygen uptakes more rapid than 500 μl./hour, the rate of shaking of the manometers was increased from 120 to 175 oscillations/min.

Preparations of enzymes. The method of preparation is described in the appropriate section for each enzyme. In general standard methods were used, though new methods were developed for preparing the lactic, glucose, succinic, malic and α-glycerophosphoric dehydrogenases. A high speed ball mill was found to offer a successful means of extracting enzymes from yeast[1]. We are

[1] The problem of extracting enzymes from micro-organisms by the method of fine grinding is now being studied *in extenso* by one of us (D. E. G.) in collaboration with V. H. Booth.

indebted to J. W. Barnard of the National Institute for Medical Research for the loan of this ingenious steel ball mill, devised by Barnard and Hewlett [1911]. Cakes of Delft or English baker's yeast were plasmolysed with sodium chloride and ground in the ball mill for 15 min. at 1500 r.p.m. The charge of yeast was usually 20–25 ml. The suspension was removed from the mill and centrifuged hard for 30 min. The viscous yellow fluid was carefully decanted and mixed with three volumes of saturated ammonium sulphate. The fine precipitate was filtered, collected on hardened paper and thoroughly dried by pressing out the moisture. The precipitate was then dissolved in about 20 ml. of phosphate buffer p_H 7·2. Methylene blue experiments disclosed the presence of fairly active lactic, α-glycerophosphoric, hexosemonophosphoric and hexosediphosphoric dehydrogenases as shown by Table I.

Table I.

Enzyme ml.	Methylene blue. 1/5000 ml.	Cozymase 400 units/ml. ml.	Substrate. 1 ml. of $M/10$	Time in min.
1	1	0·5	—	>30
1	1	0·5	Lactate	1
1	1	0·5	Hexosemonophosphate	4
1	1	0·5	Hexosediphosphate	4
1	1		α-Glycerophosphate	7

The lactic enzyme is particularly active. In some cases 1 ml. of the buffered enzyme solution took up as much as 2000 μl. oxygen in one hour in presence of lactate and some suitable carrier. It is also interesting to note that the extract of ground yeast contains the indophenol oxidase. The oxidase can oxidise *p*-phenylenediamine to the characteristic blue oxidation product but takes up oxygen slowly in presence of *p*-phenylenediamine.

Coenzymes. Cozymase was prepared by the method of Myrbäck [1933]. The purification process was carried as far as the precipitation with mercuric nitrate. The strength of the coenzyme solution was determined by comparing the rate of reduction of methylene blue by the glucose dehydrogenase system in presence of graded dilutions of the unknown solutions with the rate of reduction in presence of graded dilutions of a standard cozymase solution kindly supplied to us by Dr Myrbäck. Since the activity of the glucose dehydrogenase is proportional to the concentration of cozymase within fairly wide limits, the method is capable of a high degree of accuracy.

The hexosemonophosphate coenzyme was prepared from horse blood by the method of Warburg and Christian [1932, 2].

The lactic coenzyme from heart was prepared by the method of Banga *et al.* [1932]. When tested with various dehydrogenase systems, it failed to yield as satisfactory results as cozymase. Probably our cozymase solutions were more concentrated than were the solutions of lactic coferment. The comparison therefore of relative activities may not be valid.

Intermediary carriers. Cytochrome *c* was prepared by the method of Keilin [1930]. There is a tendency for cytochrome in the form of the acid haematin to go into solution when being washed after SO_2 precipitation. It was found that washing with 1 % trichloroacetic acid rather than distilled water checked the tendency of precipitated cytochrome to dissolve and permitted very thorough extraction of all water-soluble impurities. The strengths of the cytochrome solutions were determined by colorimetric comparison with standard solutions of pyridine-mesohaemochromogen, the absorption spectrum of which is very similar

to that of cytochrome. Since cytochrome solutions tend to deteriorate on standing for more than a fortnight even at 0°, the bulk of the precipitated cytochrome was left suspended in 1 % trichloroacetic acid and dissolved in alkali when required.

Oxidised glutathione was prepared by the method of Pirie [1931]. 400 mg. were dissolved in 25 ml. of distilled water, the free acid was neutralised with NaOH and the volume made up to 100 ml.

Solutions of purified lactoflavin were obtained from the I.G. Farbenindustrie through the courtesy of Prof. P. György. The solutions, which contained 1 mg. in 2 ml., were diluted to yield a final concentration of 100γ lactoflavin per ml. A sample of synthetic ribose-flavin in a concentration of 10γ per ml. was also used for comparison purposes.

We are indebted to Prof. O. Warburg for his generous gifts of yellow pigment in the form of the crude and the purified preparations. The former was used as a 20 % solution, the latter as a 5 % solution, both of which are equivalent to 15γ lactoflavin per ml.

Results.

Succinic dehydrogenase and indophenol oxidase. These two enzymes are invariably concomitant in extracts of animal tissues, and the following methods of preparation apply to both. Ox or sheep heart was minced, washed with 20 volumes of tap water at least 5 times and then ground with fine quartz sand in a mechanical mortar for 30 min. The resulting pulp was squeezed through muslin. The haemoglobin-rich liquid was brought to p_H 4·6 with acetic acid and centrifuged for 10 min. The supernatant fluid was discarded and the colourless sediment was suspended in phosphate buffer p_H 7·2. The enzyme "solution" may be kept for a week at 0° without serious loss of activity.

The enzyme "solution" prepared from ox heart contains an indophenol oxidase which is about twice as active as the succinoxidase whereas the enzyme "solution" prepared from sheep heart contains the two enzymes in approximately the same strengths (see Table IV).

A succinoxidase was also prepared from ox liver. The fresh tissue was minced in a Latapie mincer. The pulp was mixed with 3 volumes of Ringer's solution and allowed to stand 30 min. before centrifuging. The supernatant fluid was mixed with 2 volumes of saturated ammonium sulphate solution and the precipitate filtered off. The precipitate was resuspended in saturated ammonium sulphate solution and filtered until dry on a Büchner funnel. The precipitate was then dissolved in phosphate buffer p_H 7·2.

The potentials of the succinic-fumaric system are about 200 mv. more positive than those of flavin, yellow pigment and glutathione. Hence cytochrome alone of all the known intermediary carriers is thermodynamically capable of reduction by the succinoxidase system [Lehmann, 1929; Borsook and Schott, 1931; Kuhn and Moruzzi, 1934; Stern, 1934; Bierich *et al.*, 1934; Green, 1933; 1934].

Table II. *The effect of KCN on the catalysis of the succinoxidase of ox heart by methylene blue and cytochrome.*

Enzyme (ml.)	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Succinate, 10 %	—	1	1	1	1	1	1	—
Methylene blue, 1 %	—	—	0·2	0·2	—	—	—	—
KCN, $M/20$	—	—	—	0·1	0·1	—	0·1	—
Cytochrome, 3×10^{-4} M	—	—	—	—	—	0·5	0·5	0·5
Distilled water	2·5	1·5	1·3	1·2	1·4	1	0·9	2
O_2 uptake (μl./30 min.)	0	230	325	279	35·3	825	46·1	16·0
% increase	—	—	41	21	—	260	—	—

Table III. *The effect of varying cytochrome concentrations on the succinoxidase of ox heart.*

Enzyme (ml.)	0·5	0·5	0·5	0·5	0·5
Succinate, 10%	0·5	0·5	0·5	0·5	0·5
Cytochrome, 3×10^{-4} *M*	—	0·5	—	—	—
Cytochrome, diluted $\frac{1}{2}$	—	—	0·5	—	—
Cytochrome, diluted $\frac{1}{4}$	—	—	—	0·5	—
Cytochrome, diluted $\frac{1}{16}$	—	—	—	—	0·5
Distilled water	2	1·5	1·5	1·5	1·5
O_2 uptake (μl./30 min.)	106	436	339	304	160
% increase	—	310	220	187	51

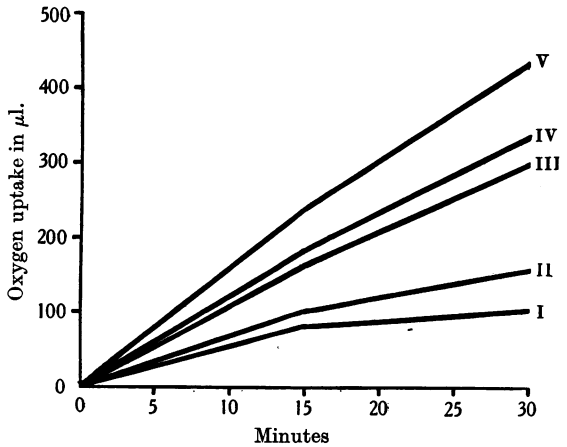

Fig. 1. The effect of varying cytochrome concentrations on the oxygen uptake of the succinoxidase of ox heart. Concentration of cytochrome 3×10^{-4} *M*. I, no cytochrome; II, diluted $\frac{1}{16}$; III, diluted $\frac{1}{4}$; IV, diluted $\frac{1}{2}$; V, undiluted.

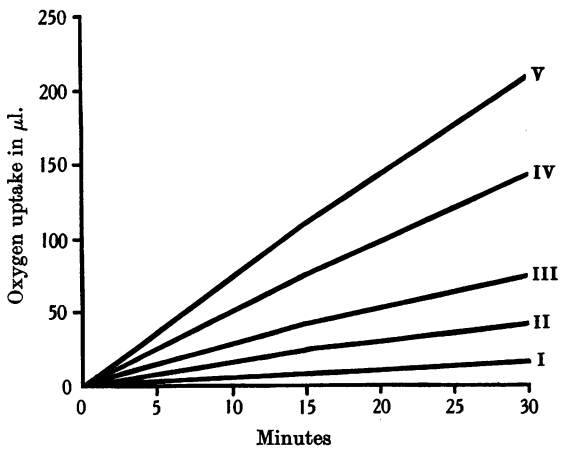

Fig. 2. The effect of varying cytochrome concentrations on the oxygen uptake of the succinoxidase of liver. Concentration of cytochrome 3×10^{-4} *M*. I, no cytochrome; II, diluted $\frac{1}{16}$; III, diluted $\frac{1}{8}$; IV, diluted $\frac{1}{2}$; V, undiluted.

Tables II and III and Fig. 1 show the effects of cytochrome and methylene blue on the oxygen uptake of ox heart succinoxidase. Methylene blue produces an increase of 40 % as compared with an increase of 260 % with cytochrome. It is interesting to note that cyanide in a final concentration of $M/600$ has very little effect on the methylene blue catalysis although completely inhibiting the cytochrome catalysis. In Table IV there is a comparison of the effects of cytochrome

Table IV. *Comparison of the indophenol oxidase and succinoxidase activities of ox and sheep heart preparations.*

Enzyme, ox heart (ml.)	0·5	0·5	0·5	—	—	—
Enzyme, sheep heart (ml.)	—	—	—	0·5	0·5	0·5
Succinate, 10 %	—	1·0	1·0	—	1·0	1·0
p-Phenylenediamine, 10 mg./ml.	0·5	—	—	0·5	—	—
Cytochrome, 3×10^{-4} *M*	—	—	1·0	—	—	1·0
Distilled water	2	1·5	0·5	2	1·5	0·5
O_2 uptake (μl./30 min.)	225	116	637	170	175	316
% increase	—	—	450	—	—	81

on the succinoxidase of ox and sheep heart. The increase with the former enzyme is 450 % and with the latter only 81 %. This difference in the magnitude of the effect has been observed repeatedly.

The liver succinoxidase takes up practically no oxygen in presence of the substrate alone. Tables V and VI and Fig. 2 show the effect of cytochrome on the

Table V. *The effect of cytochrome on the liver succinoxidase.*

Enzyme (ml.)	1	1	1	1
Succinate, 10 %	—	0·5	0·5	—
p-Phenylenediamine, 10 mg./ml.	—	—	—	0·5
Cytochrome, 3×10^{-4} *M*	—	—	0·5	—
Distilled water	2	1·5	1	1·5
O_2 uptake (μl./30 min.)	0	22·5	111	32
% increase	—	—	390	—

Table VI. *The effect of varying cytochrome concentrations on the succinoxidase of liver.*

Enzyme (ml.)	1	1	1	1	1	1	1
Succinate, 10 %	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Cytochrome, 3×10^{-4} *M*	—	1	—	—	—	—	—
Cytochrome, diluted $\frac{1}{2}$	—	—	1	—	—	—	—
Cytochrome, diluted $\frac{1}{3}$	—	—	—	1	—	—	—
Cytochrome, diluted $\frac{1}{4}$	—	—	—	—	1	—	—
Cytochrome, diluted $\frac{1}{8}$	—	—	—	—	—	1	—
Cytochrome, diluted $\frac{1}{16}$	—	—	—	—	—	—	1
Distilled water	1·5	0·5	0·5	0·5	0·5	0·5	0·5
O_2 uptake (μl./30 min.)	16	205	145	128	91	73·5	40
% increase	—	1180	810	700	470	360	150

oxygen uptake. The increase in velocity is proportional to the concentration of added cytochrome. It is noteworthy that although the activity of the indophenol oxidase as measured by the oxidation of *p*-phenylenediamine is very low, nevertheless a large cytochrome effect is obtained. Since the indophenol oxidase is directly concerned in the oxidation of cytochrome, the question arises how an effect of the magnitude observed with the liver preparation can be accounted for on the basis of a very feeble indophenol oxidase. The explanation lies in the fact that the activity of the indophenol oxidase as measured by the oxidation of *p*-phenylenediamine seems to depend upon the presence of cytochrome. Indeed if cytochrome is added to any indophenol oxidase preparation, there is invariably a large increase in the velocity of the oxygen uptake. Spectroscopically

it can also be shown that *p*-phenylenediamine reduces cytochrome directly. Thus it may be that *p*-phenylenediamine is not acted upon by the enzyme directly except through the intermediation of cytochrome. That is to say *p*-phenylenediamine reduces cytochrome, and reduced cytochrome is then oxidised under the influence of the indophenol oxidase.

The experiments described offer clear proof (1) that the cytochrome-indophenol oxidase system catalyses the reaction of succinic acid with molecular oxygen, and (2) that when there is little cytochrome present, as in the succin-oxidase of liver, there is no oxidation of succinic acid. The large blank in the ox and sheep heart preparations is probably due to the fairly high concentrations of cytochrome *a*, *b* and *c* already present. The greater magnitude of the cytochrome effect in the case of ox heart succinoxidase is likewise probably due to the presence of less cytochrome than in the succinoxidase of sheep heart.

It has been shown by Dixon [1927] that although the oxidation of sodium succinate is inhibited by cyanide it is not inhibited by CO which inhibits the indophenol oxidase. Keilin [1929] suggested the explanation that the indophenol oxidase, being in much higher concentration than succinoxidase, is never working at maximum velocity. Consequently the partial inhibition of the oxidase by CO cannot be detected by changes in the oxygen uptake of the preparation.

Lactic dehydrogenase. The enzyme was prepared from fresh yeast by the method of ball mill grinding described above. The dehydrogenase in presence of lactate will reduce methylene blue without addition of cozymase. However, the addition of cozymase usually increases the speed of reduction considerably. Furthermore, in oxygen experiments there is a tendency for the activity of the enzyme to fall off rapidly with time when no coenzyme is provided. It appears therefore that the lactic enzyme of yeast is quite normal with respect to its dependence upon a coenzyme and the incomplete dependence may be due to the fact that the coenzyme is either chemically bound or adsorbed in such a way as to resist washing out.

Table VII shows the effects of various carriers on the oxygen uptake of the lactic dehydrogenase. Only methylene blue and cytochrome are at all effective

Table VII. *The effects of methylene blue and natural carriers on the lactic dehydrogenase of yeast.*

Enzyme (ml.)	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Lactate, 10%	—	0·5	0·5	0·5	0·5	0·5	—	0·5	0·5	0·5	0·5	0·5
Cozymase, 10 units/ml.	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Methylene blue, 1%	—	—	0·2	0·2	—	—	—	—	—	—	—	—
KCN, *M*/20	—	—	—	0·1	—	—	—	—	0·1	—	—	—
Cytochrome, 3×10^{-4} *M*	—	—	—	—	0·5	0·5	0·5	—	0·5	—	—	—
Indophenol oxidase	—	—	—	—	—	0·5	0·5	0·5	0·5	—	—	—
Yellow pigment, 5%	—	—	—	—	—	—	—	—	—	0·5	—	—
Flavin, 100 γ/ml.	—	—	—	—	—	—	—	—	—	—	0·5	—
Glutathione, 100 mg. %	—	—	—	—	—	—	—	—	—	—	—	0·5
Distilled water	2	1·5	1·3	1·2	1	0·5	1	1	0·4	1	1	1
O_2 uptake (μl./30 min.)	19·7	68	361	362	149	412	20·6	64·5	41	66	51	56
% increase	—	—	430	430	120	507	—	—	—	—	—	—

as respiratory carriers, the former producing an increase in the oxygen uptake of 430% and the latter an increase of 507%. *M*/600 cyanide has no effect whatsoever on the methylene blue catalysis but completely poisons the cytochrome catalysis. The addition of cytochrome without oxidase does increase the oxygen uptake from 68 μl. to 149 μl., but it must be remembered that the

extracts of ground yeast contain a certain amount of indophenol oxidase although very small in comparison with the lactic enzyme. Reducing the concentration of cytochrome to one-quarter reduces the cytochrome oxidase effect almost by one-half. Table VIII and Fig. 3 show that the cytochrome effect is sustained with time.

Table VIII. *The effect of varying cytochrome concentrations on the lactic dehydrogenase of yeast.*

Enzyme (ml.)	0·5	0·5	0·5	0·5
Cozymase, 10 units/ml.	0·5	0·5	0·5	0·5
Lactate, 10%	0·5	0·5	0·5	0·5
Indophenol oxidase	—	0·5	0·5	0·5
Cytochrome, $3 \times 10^{-4} M$	—	1	0·5	0·25
Distilled water	1·5	0	0·5	0·75
O_2 uptake (μl./30 min.)	68	430	412	259
% increase	—	535	507	280

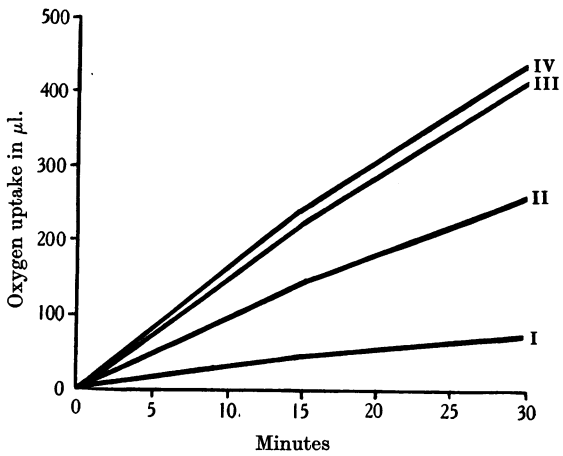

Fig. 3. The effect of varying cytochrome concentrations on the oxygen uptake of the yeast lactic dehydrogenase. Concentration of cytochrome $3 \times 10^{-4} M$. I, no cytochrome; II, 0·25 ml. cytochrome; III, 0·5 ml. cytochrome; IV, 1 ml. cytochrome.

α-Glycerophosphate dehydrogenase. The enzyme was prepared from Delft yeast by the method of grinding in the ball mill, and from the skeletal muscles of rabbit by the following method. The muscles dissected from the hind legs of a freshly killed rabbit were minced in a Latapie mincer. The pulp was washed several times with Ringer's solution, ground with quartz sand for 30 min. and suspended in $M/4$ phosphate buffer p_H 7·2. The grinding must be very efficient in order to obtain a sufficiently homogeneous suspension which can be easily pipetted. Numerous attempts were made to extract the enzyme from the tissue but none was successful. It appears that the α-glycerophosphate dehydrogenase is closely associated with tissue particles and cannot be brought into solution. The enzyme is specific for the α-form of glycerophosphoric acid. The product of oxidation is as yet unknown. Presumably it is glyceraldehydephosphoric acid. Both the yeast and the animal enzyme can reduce methylene blue or oxygen in presence of α-glycerophosphate. No coenzyme is required. The addition of cozymase or any other coferment has no effect on the velocity of oxidation.

Tables IX, X, XI and XII contain the pertinent data for the α-glycerophosphate enzymes of yeast and muscle. The small increases occasioned by methylene blue rather indicate that the enzymes are both aerobic oxidases. The

Table IX. *The effects of methylene blue and intermediary carriers on the α-glycerophosphate dehydrogenase of rabbit muscle.*

Enzyme (ml.)	1	1	1	1	1
α-Glycerophosphate, *M*/10	—	0·5	0·5	0·5	0·5
Methylene blue, 1%	—	—	0·2	—	—
Cytochrome, $3 \times 10^{-4} M$	—	—	—	0·5	—
Yellow pigment, 5%	—	—	—	—	0·5
Distilled water	2	1·5	1·3	1	1
O_2 uptake (μl./30 min.)	10	115	157	38	116
% increase	—	—	36·5	—	—

Table X. *The effects of KCN and glutathione on the α-glycerophosphate dehydrogenase of rabbit muscle.*

Enzyme (ml.)	1	1	1
α-Glycerophosphate, *M*/10	0·5	0·5	0·5
KCN, *M*/100	—	0·5	—
Glutathione (GSSG), 200 mg. %	—	—	0·5
Distilled water	1·5	1	1
O_2 uptake (μl./30 min.)	95	75	105
% increase	—	—	10·5

Table XI. *The effects of KCN (final concentration M/600) on the α-glycerophosphate dehydrogenase of yeast.*

Enzyme (ml.)	1	1
α-Glycerophosphate, *M*/10	0·5	0·5
KCN, *M*/100	—	0·5
Distilled water	1·5	1
O_2 uptake (μl./30 min.)	104	59

Table XII. *The effects of methylene blue and intermediary carriers on the α-glycerophosphate dehydrogenase of yeast.*

Enzyme (ml.)	1	1	1	1	1	1
α-Glycerophosphate, *M*/10	—	0·5	0·5	0·5	0·5	0·5
Methylene blue, 1%	—	—	0·2	—	—	—
Cytochrome, $3 \times 10^{-4} M$	—	—	—	0·5	—	—
Indophenol oxidase	—	—	—	0·5	—	—
Yellow pigment, 5%	—	—	—	—	0·5	—
Flavin, 100 γ/ml.	—	—	—	—	—	0·5
Distilled water	2	1·5	1·3	0·5	1	1
O_2 uptake (μl./30 min.)	9	96	100	84	57	66
% increase	—	—	4·2	—	—	—

ability to react directly with oxygen can never be eliminated by any amount of washing, precipitation *etc.* *M*/600 KCN inhibits the enzyme of rabbit muscle 20%, and the enzyme of yeast 40%. Cytochrome, yellow pigment and flavin have, if anything, inhibitory effects on the velocity of oxygen uptake. Glutathione, in several experiments, was found to increase the oxygen uptake slightly but definitely although there was no evidence that glutathione could be reduced anaerobically by the α-glycerophosphate system.

Formic dehydrogenase. The enzyme was prepared from *Bact. coli* by the method of Stickland [1929] and stored at 0°.

Table XIII shows the effect of intermediary carriers on the oxygen uptake. The blank of the preparation in absence of formate is rather high (50 μl. in

Table XIII. *The effect of methylene blue and intermediary carriers on the formic dehydrogenase from* Bact. coli.

Enzyme (ml.)	1	1	1	1	1	1	1	1	1	1
Formate, 10%	—	1	1	1	1	1	1	1	1	—
Methylene blue, 1%	—	—	—	0·2	0·2	—	—	—	—	—
KCN, *M*/20	—	—	0·1	—	0·1	—	—	—	—	—
Yellow pigment, 5%	—	—	—	—	—	0·5	—	—	—	—
Flavin, 100γ/ml.	—	—	—	—	—	—	0·5	—	—	—
Glutathione, 100 mg. %	—	—	—	—	—	—	—	0·5	—	—
Cytochrome, 3×10^{-4} *M*	—	—	—	—	—	—	—	—	0·5	0·5
Indophenol oxidase	—	—	—	—	—	—	—	—	0·5	0·5
Distilled water	2	1	0·9	0·8	0·7	0·5	0·5	0·5	0	1
O_2 uptake (μl./45 min.)	49·5	57	41	161	106	80·5	69	57	97	88·5

45 min.) and the addition of formate hardly increases this value. Methylene blue increases the rate of formate oxidation about fifteenfold. *M*/600 KCN inhibits the methylene blue catalysis by about 50%. Flavin, glutathione and cytochrome have very small effects on the oxygen uptake. Little significance can be attached to effects of such magnitude. The effect of yellow pigment is definite though small. It should be pointed out that there is no effect of these carriers on the blank reaction, and that the effect is entirely on the oxidation of formate.

The formic enzyme solution contains the entire formic activity of the bacterial suspension used in the preparation [Stickland, 1929] as measured by methylene blue reduction. It was of interest therefore to compare the reaction with molecular oxygen of a given amount of formic enzyme with the equivalent amount of enzyme in the intact bacterial cells. Table XIV shows this comparison.

Table XIV. *Comparison of the activities of* Bact. coli, *toluene treated* Bact. coli, *and formic dehydrogenase from* Bact. coli.

Enzyme (ml.)	1	1	—	—	—	—
Bact. coli (ml.)	—	—	1	1	—	—
Bact. coli (toluene-treated) (ml.)	—	—	—	—	1	1
Formate, 10%	1	1	—	1	—	1
Methylene blue, 1%	—	0·2	—	—	—	—
Distilled water	1	0·8	2	1	2	1
O_2 uptake (μl./30 min.)	57	161	65	194	0	123·5

Methylene blue enables the cell-free enzyme to react with oxygen at the same speed as that in presence of the natural carrier *in vivo*. The table offers another proof that neither yellow pigment, flavin, cytochrome nor glutathione can be identical with the natural carrier for the formic enzyme in *Bact. coli*.

Hexosemonophosphate dehydrogenase. The enzyme was obtained from three sources: (1) horse corpuscles washed thrice with 0·9% NaCl and laked with distilled water, (2) dialysed yeast juice, and (3) the ammonium sulphate precipitate of dialysed yeast juice. Considerable difficulty was encountered in the preparation of the enzyme from maceration extract of bottom yeast by the method of Warburg and Christian [1932, 2], the final preparations being rather inactive. None of the preparations from the above three sources attained the activity recorded by Warburg and Christian. However, the activities were sufficiently high to test the action of intermediary carriers. The coferment from horse corpuscles was used in all the experiments quoted below. Euler and Adler [1934] have claimed that cozymase can replace the Warburg coferment in the hexosemonophosphate system. Our experience has been that, in aerobic experiments, the two coferments are not equivalent. A sample of cozymase (400 Co units per ml.) which enabled the glucose system to work at maximum velocity

was hardly as efficient as crude Warburg coferment in the hexosemonophosphate system.

Table XV shows the effects of methylene blue, yellow pigment, flavin, glutathione and cytochrome on the aerobic oxidation of hexosemonophosphate by the enzyme of horse corpuscles. Yellow pigment and methylene blue are extremely active, in accordance with the findings of Warburg and Christian [1932, 2], but flavin, glutathione and cytochrome are inactive.

Table XV. *The effects of methylene blue and intermediary carriers on the hexosemonophosphate dehydrogenase of horse corpuscles.*

Enzyme (ml.)	1	1	1	1	1	1	1
Warburg coferment	1	1	1	1	1	1	1
Hexosemonophosphate 0·35 *M*	0·2	0·2	0·2	—	0·2	0·2	0·2
Methylene blue, 0·1%	—	0·5	—	—	—	—	—
Cytochrome, 3×10^{-4} *M*	—	—	0·4	0·4	—	—	—
Indophenol oxidase	—	—	0·3	0·3	—	—	—
Yellow pigment, 5%	—	—	—	—	0·3	—	—
Flavin, 20γ/ml.	—	—	—	—	—	0·3	—
Glutathione, 400 mg. %	—	—	—	—	—	—	0·5
Distilled water	0·8	0·3	0·1	0·3	0·5	0·5	0·3
O_2 uptake (μl./30 min.)	14	292	0	0	157	21	15

In experiments with the hexosemonophosphate enzyme from the two other sources, *viz.* dialysed yeast juice and the ammonium sulphate precipitate of yeast juice, yellow pigment was again found to be the only natural carrier with positive effect.

Meldrum and Tarr [1935] have found that the hexosemonophosphate system can reduce glutathione either aerobically or anaerobically. The inability of glutathione to act as an aerobic carrier must therefore be due to its slowness of autoxidation under the experimental conditions obtaining.

Hexosediphosphate dehydrogenase. It is generally assumed that the same dehydrogenase activates both hexosemonophosphate and hexosediphosphate. There are three lines of evidence in favour of this view. (1) The two enzymes always accompany one another. (2) The Warburg coferment from horse corpuscles is required by both enzymes. (3) The reactions of the two enzymes towards yellow pigment, methylene blue, KCN *etc.*, are more or less similar. While investigating the possibility of obtaining a hexosemonophosphate enzyme from sources other than yeast or red blood cells, we observed that the ratio

$$\frac{\text{activity towards hexosemonophosphate}}{\text{activity towards hexosediphosphate}}$$

varied widely. In some cases the hexosediphosphate system was far more active, in other cases the reverse was true. The question of the identity of these two enzymes requires therefore further investigation.

Enzymes can be prepared from liver, heart and other tissues which will reduce methylene blue fairly rapidly in presence of Warburg coferment and hexosediphosphate. Unfortunately the reaction of these enzymes with molecular oxygen in presence of methylene blue or yellow pigment is feeble and falls off rapidly with time. Horse corpuscles were found to offer the most satisfactory source of the enzyme.

Table XVI contains the data for the hexosediphosphate system. Yellow pigment produces the greatest increase (220%). Methylene blue and glutathione are somewhat less effective. The flavin catalysis is small. The cytochrome control experiment shows that the cytochrome-indophenol oxidase system has no effect on the oxygen uptake.

Table XVI. *The effects of methylene blue and intermediary carriers on the hexosediphosphate dehydrogenase.*

Enzyme (ml.)	1·5	1·5	1·5	1·5	1·5	1·5	1·5
Warburg coferment	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Hexosediphosphate $M/3$	0·3	0·3	0·3	—	0·3	0·3	0·3
Methylene blue, 0·1%	—	0·5	—	—	—	—	—
Yellow pigment, 5%	—	—	—	—	0·5	—	—
Flavin, 20γ/ml.	—	—	—	—	—	0·5	—
Glutathione, 400 mg. %	—	—	—	—	—	—	0·5
Cytochrome, 3×10^{-4} M	—	—	0·3	0·3	—	—	—
Indophenol oxidase	—	—	0·4	0·4	—	—	—
Distilled water	0·7	0·2	0	0·3	0·2	0·2	0·2
O_2 uptake (μl./30 min.)	26·5	67·5	34·4	19·4	83·5	45·0	72·5
% increase	—	158	—	—	220	73	177

Glucose dehydrogenase. The enzyme was prepared from liver (1) by the standard method of Harrison [1933] and (2) by our own method. Finely minced liver was dried *in vacuo*, ground to a fine powder and suspended in water for 30 min. The murky solution was centrifuged and the precipitate discarded. The supernatant fluid was dialysed for 2 days against distilled water (4 changes) and then fully saturated with ammonium sulphate. The precipitate was filtered off and dried *in vacuo*. The preparation can be kept for several months at 0° without much loss of activity. The enzyme was prepared for use by grinding this liver powder with phosphate buffer p_H 7·2 and centrifuging down insoluble material.

Cozymase was used in preference to the Harrison coenzyme since it can be prepared in a comparatively pure and concentrated state.

Tables XVII, XVIII, XIX show the effects of the various carriers on the oxidation of glucose by the enzyme of dried liver. Methylene blue increases the oxygen uptake two- or three-fold and $M/600$ KCN augments this catalysis. Flavin and cytochrome have practically no effect. Yellow pigment and glutathione both produce definite increases. Table XX shows a more pronounced yellow pigment effect with another preparation of dried liver. The magnitude of

Table XVII. *The effects of methylene blue and cytochrome on the glucose dehydrogenase.*

Enzyme (ml.)	1	1	1	1
Cozymase, 83 units/ml.	0·5	0·5	0·5	0·5
Glucose, 3 M	0·5	0·5	0·5	—
Methylene blue, 0·1%	—	0·5	—	—
Cytochrome, 3×10^{-4} M	—	—	0·5	0·5
Indophenol oxidase	—	—	0·5	0·5
Distilled water	1	0·5	0	0·5
O_2 uptake (μl./30 min.)	40	138	40·7	30
% increase	—	256	—	—

Table XVIII. *The effects of intermediary carriers and cyanide on the glucose dehydrogenase.*

Enzyme (ml.)	1	1	1	1	1	1	1
Cozymase, 400 units/ml.	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Glucose, 3 M	—	0·5	0·5	0·5	0·5	0·5	0·5
Methylene blue, 0·1%	—	—	0·5	0·5	—	—	—
KCN, $M/100$	—	—	—	0·5	—	—	—
Yellow pigment, 5%	—	—	—	—	0·5	—	0·5
Flavin, 10γ/ml.	—	—	—	—	—	0·5	0·5
Distilled water	1·5	1	0·5	0	0·5	0·5	0
O_2 uptake (μl./30 min.)	63	128	391	417	146	134	162
% increase	—	—	206	226	—	—	27

Table XIX. *The effect of methylene blue and intermediary carriers on the glucose dehydrogenase.*

Enzyme (ml.)	1	1	1	1
Cozymase, 83 units/ml.	0·5	0·5	0·5	0·5
Glucose, 3 *M*	0·5	0·5	0·5	0·5
Methylene blue, 0·1 %	—	0·5	—	—
Flavin, 20γ/ml.	—	—	0·5	—
Glutathione, 400 mg. %	—	—	—	0·5
Distilled water	1	0·5	0·5	0·5
O_2 uptake (μl./30 min.)	78	330	88	96
% increase	—	324	—	23

Table XX. *The effect of yellow pigment on the glucose dehydrogenase.*

Enzyme (ml.)	0·5	0·5
Cozymase, 400 units/ml.	0·5	0·5
Glucose, 3*M*	0·3	0·3
Yellow pigment, 20%	—	0·5
Distilled water	1·7	1·2
O_2 uptake (μl./30 min.)	102	204
% increase	—	100

the effect seems to be somewhat variable and dependent upon the particular preparation.

Adler and Euler [1935] have already observed the increase of the glucose oxidation by yellow pigment. Their theoretical conclusions from this observation will be discussed elsewhere.

Harrison [1931] produced evidence that the cytochrome-indophenol oxidase system catalyses the reaction of glucose with molecular oxygen in presence of the enzyme. We have been unable to confirm his results using various preparations of the glucose enzyme, of cozymase, of the glucose coenzyme, of cytochrome and of the indophenol oxidase. Tables XXI and XXII compare the actions of the cytochrome-indophenol oxidase system on the Harrison enzyme and on the dried liver preparation. If anything the cytochrome-indophenol oxidase system is inhibitory. It should be mentioned that in all experiments involving the

Table XXI. *The effects of cytochrome and indophenol oxidase on the glucose dehydrogenase.* (*Harrison enzyme.*)

Enzyme (ml.)	0·5	0·5	0·5	0·5	0·5	0·5
Cozymase, 400 units/ml.	0·5	0·5	0·5	0·5	0·5	0·5
Glucose, 3 *M*	—	0·3	0·3	—	0·3	—
*Cytochrome (1), 3×10^{-4} *M*	—	—	1	1	—	—
*Cytochrome (2), 3×10^{-4} *M*	—	—	—	—	1	1
Indophenol oxidase	—	—	0·7	0·7	0·7	0·7
Distilled water	2	1·7	0	0·3	0	0·3
O_2 uptake (μl./30 min.)	23·5	49·4	32·5	29·1	35	39

* Cytochrome (1), fresh. Cytochrome (2), old stock.

Table XXII. *The effects of cytochrome and indophenol oxidase on the glucose dehydrogenase.* (*Dried liver enzyme.*)

Enzyme (ml.)	0·5	0·5	0·5	0·5	0·5	0·5
Cozymase, 400 units/ml.	0·5	0·5	0·5	0·5	0·5	0·5
Glucose, 3 *M*	—	0·3	0·3	—	0·3	—
*Cytochrome (1), 3×10^{-4} *M*	—	—	1	1	—	—
*Cytochrome (2), 3×10^{-4} *M*	—	—	—	—	1	1
Indophenol oxidase	—	—	0·7	0·7	0·7	0·7
Distilled water	2	1·7	0	0·3	0	0·3
O_2 uptake (μl./30 min.)	46·5	98	70·6	38·4	86	34·4

* Cytochrome (1), fresh. Cytochrome (2), old stock.

addition of cytochrome and the oxidase, these components had been previously tested for activity with the succinoxidase or lactic enzyme, and unless an increase in the oxygen uptake of several hundred per cent. was obtained, they were not used with other systems. There have been cases in which the addition of cytochrome and indophenol oxidase increased the oxygen uptake two- or three-fold, but in all such cases appropriate controls disclosed (1) that the effect was to a great extent independent of the presence of glucose and (2) that the total effect was the sum of the individual effects of cytochrome or oxidase.

Mann [1932] observed the anaerobic reduction of oxidised glutathione by the glucose system. The relative inefficiency of glutathione as an aerobic carrier in this system once again must be referred to the sluggish autoxidation of the reduced form under the conditions of the experiment.

Aerobic oxidases.

This class of oxidising enzymes is capable of reacting directly with molecular oxygen in the complete absence of intermediary carrier. Some of the aerobic oxidases, like xanthine oxidase, reduce methylene blue or any other suitable hydrogen acceptor; the rest, like the urico-oxidase, are specific for molecular oxygen. It was considered of interest to test the effect of natural carriers on the oxygen uptake of three representative aerobic oxidases—xanthine, urico- and amino-acid oxidases.

Xanthine oxidase. The enzyme was prepared from whey by the method of Dixon and Kodama [1926]. The dried powder was dissolved in phosphate buffer p_H 7·2 and used directly. Table XXIII contains the data for the xanthine

Table XXIII. *The effects of methylene blue and intermediary carriers on the xanthine oxidase.*

Enzyme (ml.)	1·5	1·5	1·5	1·5	1·5	1·5	1·5
Hypoxanthine, $M/10$	—	0·3	0·3	0·3	0·3	0·3	0·3
Methylene blue, 0·1%	—	—	0·5	—	—	—	—
Cytochrome, $3 \times 10^{-4} M$	—	—	—	0·5	—	—	—
Indophenol oxidase	—	—	—	0·5	—	—	—
Yellow pigment, 5%	—	—	—	—	0·5	—	—
Flavin, 100γ/ml.	—	—	—	—	—	0·5	—
Glutathione, 400 mg./100 ml.	—	—	—	—	—	—	0·5
Distilled water	1·5	1·2	0·7	0·2	0·7	0·7	0·7
O_2 uptake, μl./30 min.)	9	93·5	116	104	108	23·2	80
% increase	—	—	23·4	10·6	15	—	—

oxidase system. Methylene blue increases the oxygen uptake 25%. All the other positive effects are too small to merit any attention. Flavin in the concentration used exerts a strong inhibition. Green and Dixon [1934] have shown that the flavin present in milk cannot account for the reaction of xanthine oxidase with molecular oxygen.

Urico-oxidase. The enzyme was prepared from pig liver by extracting with acetone, pulverising the dried residue and suspending 1 g. in 10 ml. of borate buffer p_H 8·5. The uric acid was suspended in buffer of the same p_H in a concentration of 10 mg. per ml. As the oxidation proceeds, the suspended uric acid gradually goes into solution. Table XXIV shows that none of the natural intermediary carriers has any effect.

Amino-acid oxidase. The enzyme was prepared from pig kidney by the method of Krebs [1935]. Table XXV shows that the oxidation of alanine is not affected appreciably by any of the natural carriers. $M/600$ KCN has no inhibitory effect, in agreement with the findings of Krebs [1933].

Table XXIV. *The effects of intermediary carriers on the urico-oxidase (2 Exps.).*

Enzyme (ml.)	2	2	2	2	2	1	1	1
Uric acid, 10 mg./ml.	—	0·5	0·5	0·5	0·5	0·5	0·5	—
Cytochrome, $3 \times 10^{-4} M$	—	—	—	—	—	—	0·5	0·5
Indophenol oxidase	—	—	—	—	—	0·5	0·5	0·5
Yellow pigment, 20%	—	—	0·5	—	—	—	—	—
Flavin, 20γ/ml.	—	—	—	0·5	—	—	—	—
Glutathione, 400 mg./100 ml.	—	—	—	—	0·5	—	—	—
Distilled water	1	0·5	0	0	0	1	0·5	1
O_2 uptake (μl./30 min.)	65	314	330	315	318	240	218	83·4

Table XXV. *The effects of methylene blue and intermediary carriers on the amino-acid oxidase (3 Exps.).*

Enzyme (p_H 8) (ml.)	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1·5	1	1	1	1
Alanine, $M/5$	—	0·5	0·5	0·5	—	0·5	0·5	—	0·5	0·5	0·5	0·5
Methylene blue, 0·1%	—	—	0·5	—	—	—	—	—	—	—	—	—
KCN, $M/100$	—	—	—	—	—	—	—	—	—	—	—	0·5
Cytochrome, $3 \times 10^{-4} M$	—	—	—	0·5	0·5	—	—	—	—	—	—	—
Indophenol oxidase	—	—	—	0·5	0·5	—	—	—	—	—	—	—
Yellow pigment, 5%	—	—	—	—	—	—	—	—	—	0·5	—	—
Flavin, 20γ/ml.	—	—	—	—	—	0·5	—	—	—	—	—	—
Glutathione, 400 mg./100 ml.	—	—	—	—	—	—	0·5	0·5	—	—	—	—
Distilled water	1·5	1	0·5	0	0·5	0·5	0·5	—	1·5	1	1·5	1
O_2 uptake (μl./30 min.)	10·5	147	146	181	40	134	189	28	236	210	296	338

Malic dehydrogenase. The method of preparation from ox heart is identical with that of the indophenol oxidase except that prolonged centrifuging is substituted for precipitation by acetic acid. The sediment is re-suspended in phosphate buffer p_H 7·2. Szent-György's lactic coferment or cozymase is required for the activity of the malic system. The enzyme is very labile indeed and the activity falls off rapidly in presence of oxygen and the substrate. Table XXVI shows that flavin and yellow pigment both increase the oxygen

Table XXVI. *The effects of intermediary carriers on the malic dehydrogenase.*

Enzyme (ml.)	1	1	1	1	1	1	1
Cozymase, 400 units/ml.	1	1	1	1	1	1	1
Malate, 1 M	—	0·5	0·5	—	0·5	0·5	0·5
Cytochrome, $3 \times 10^{-4} M$	—	—	0·5	0·5	—	—	—
Yellow pigment, 20%	—	—	—	—	0·5	—	—
Flavin, 1 mg./10 ml.	—	—	—	—	—	0·5	—
Glutathione, 400 mg./100 ml.	—	—	—	—	—	—	0·5
Distilled water	1	0·5	0	0·5	—	—	—
O_2 uptake (μl./30 min.)	14·8	38	72	37	72	114	38
% increase	—	—	—	—	90	200	—

uptake by 200 and 90% respectively. This is the first case in which flavin is more efficient than yellow pigment. The magnitude of the flavin effect depends upon the concentration. When the amount of flavin added is reduced to 10γ, the effect becomes very small. Consideration of the control experiment with cytochrome and indophenol oxidase discloses that there is no appreciable effect on the malic oxidation.

Quantitative proportions of enzymes in animal tissues and yeast.

In evaluating the quantitative rôle which a natural carrier plays in respiration, it is not only necessary to establish which of the various oxidising systems utilise this carrier but also what proportion of the total respiration these

oxidising systems constitute. Booth, Green and Ogston, in unpublished work, have attempted to estimate quantitatively the amounts of the various dehydrogenase and oxidase systems in different tissues. They have found that the most active dehydrogenase systems in practically all tissues are the succinic, hexosemonophosphoric, hexosediphosphoric and α-glycerophosphoric. The lactic and malic systems are much weaker, whilst the citric, alcohol and aldehyde systems are quantitatively negligible. There are of course certain enzymes which are very active in one particular tissue but do not have a general distribution, *e.g.* the glucose enzyme in liver, the urico-oxidase and amino-acid oxidase in liver and kidney, or the xanthine oxidase in liver.

In Part II (p. 2005), the relative strengths of different dehydrogenases in intact yeast are shown. The principal enzymes are the lactic, hexosemonophosphoric, hexosediphosphoric, α-glycerophosphoric and alcohol dehydrogenases. Whether the oxidation of glucose involves a special glucose dehydrogenase or whether the glucose becomes phosphorylated prior to oxidation is uncertain. If the first alternative is correct, then the glucose enzyme is one of the most active. In yeast the succinic, malic, formic, xanthine, citric and amino-acid enzymes have very little activity and it is doubtful whether they are even present.

Thus it appears that the important cytochrome-reducing system in animal cells is the succinic, whilst in aerobic yeast the lactic takes the place of the succinic.

We have been unable thus far to obtain active citric, alcohol and glutamic dehydrogenases. However, with the exception of the alcohol system in yeast, these dehydrogenases are quantitatively unimportant. Unfortunately the malic and hexosediphosphoric enzyme systems we have worked with were not completely satisfactory from the point of view of activity. The results obtained with these two enzymes must therefore be considered with reservations. It is hoped to verify the observed effects with more potent enzyme preparations.

Discussion.

Table XXVII summarises the results for the various dehydrogenase and oxidase systems.

The experiments with the different dehydrogenase systems disclose the extraordinary fact that of the 11 systems studied only the succinic and lactic can utilise cytochrome and indophenol oxidase for the *in vitro* reaction with molecular oxygen. How can this fact be reconciled with the general theory of Keilin?

The evidence that Keilin [1925; 1929; 1930] has adduced in favour of the view that dehydrogenases as a class react with molecular oxygen by means of cytochrome consists of the two observations, (1) that cells or tissues in anaerobiosis maintain cytochrome in the reduced state and (2) that agents like ethylurethane which inhibit the action of dehydrogenases have a similar effect on the reduction of cytochrome. When dealing with cells or tissues oxidising a variety of substrates, there is no way of deciding which particular substrate is reducing cytochrome. If, for example, yeast cells are found to reduce cytochrome in anaerobiosis, the effect may be brought about by one system, two systems or a group of systems. The spectroscopic observation of whole cells therefore can provide little information concerning the substance or substances reacting with cytochrome. Furthermore, the fact that ethyl urethane and other reagents affect dehydrogenases in the same way as they affect the *in vivo* reduction of cytochrome does not

Table XXVII.

Enzyme	Yellow pigment	Carriers: Flavin	Carriers: Cytochrome	Carriers: Glutathione	Methylene blue
Lactic	0	0	+ + + +	0	+ + + +
Hexosemonophosphoric	+ + +	0	0	0	+ + + +
Hexosediphosphoric	+ + + +	+ +	0	+ + +	+ + +
α-Glycerophosphoric	0	0	0	0	+
Formic	+	0	0	0	+ + + +
Succinic	0	0	+ + + +	0	+ + +
Xanthine	0	0	0	0	+
Uric	0	0	0	0	0
Amino-acid	0	0	0	0	0
Glucose	+ + +	0 or +	0	0 or +	+ + + +
Malic	+ +	+ + +	0	0	+ + +

0 = <25% increase
\+ = 25–50% increase
\+ + = 50–100% increase
\+ + + = 100–200% increase
\+ + + + = >200% increase

necessarily imply that all dehydrogenases must reduce cytochrome. The observation offers proof that some dehydrogenase or group of dehydrogenases is involved but the actual number of systems cannot be deduced from the observation. When Keilin [1929] washed and aerated yeast as thoroughly as possible, he found only two substrates which increased the speed of the blank reduction of cytochrome, namely lactate and glucose. If it be assumed that glucose on oxidation gives rise to lactic acid, there is complete agreement between our results and those of Keilin. That is to say the lactic is the principal and perhaps only system in yeast utilising cytochrome and the indophenol oxidase. Spectroscopic observation of washed heart muscle showed that of a large number of substrates tried only succinic acid had any appreciable effect on the rate of reduction of cytochrome. Here again our results and those of Keilin are in agreement in designating succinic acid as the principal substrate of the cytochrome system in animal tissues.

There is the possibility that the *in vivo* reduction of cytochrome by dehydrogenase systems (the succinic and lactic excepted) is not direct but involves a chain of reactions the first of which concerns the substrate and the last cytochrome. If such were the case then it would be impossible to couple dehydrogenases *in vitro* with the cytochrome-indophenol oxidase system unless the hypothetical factors concerned in the links between cytochrome and the substrate were likewise supplied. There is however no evidence for the existence of any intermediary links.

Goszy and Szent-Györgyi [1934] have suggested that the succinic-fumaric system acts as a link between the cytochrome system and substrates in the following fashion. Succinate becomes oxidised to fumarate at the expense of the reduction of cytochrome. Fumarate then reacts with some metabolite such as lactate to yield pyruvate and succinate. In this scheme only a small amount of the succinic-fumaric system is necessary to oxidise a comparatively large amount of substrate. Furthermore, the scheme allows of the entire respiration proceeding through cytochrome without the necessity of a direct reaction between cytochrome and the substrate. Green *et al.* [1934] showed that reactions between dehydrogenase systems do not proceed unless a suitable carrier is provided. The theory of Goszy and Szent-Györgyi amounts to the statement that the succinic-fumaric system can react directly with the lactic-pyruvic system in absence of a

carrier provided that the fumaric acid is supplied in the nascent state. In the experiments of Green *et al.* the fumaric acid was added directly and did not arise by oxidation. Experiments designed to test the theory are described in Part II (p. 2005). No evidence was forthcoming that the theory is correct.

Warburg *et al.* [1933] have recently suggested that oxygen reacts with the substrate through 5 links in the following order: (1) Atmungsferment (*i.e.* indophenol oxidase), (2) some unknown haematin and (3), (4) and (5) the three components of cytochrome. One of the three components of cytochrome, let us say *c*, is reduced by the substrate; *c* in turn reduces component *b etc.*, until the Atmungsferment is finally reduced. Assuming the theory to be correct, the objection may be raised to our experiments that unless the three cytochromes are all present, there is no way of testing whether a particular dehydrogenase react with the cytochrome system. It is true that only cytochrome *c* is added in the *in vitro* experiments. But the indophenol oxidase which is likewise added contains definite though small amounts of *b* and *a*, the reduced bands of which are easily seen with a hand spectroscope. Thus in all experiments the three components of cytochrome are present, albeit in disproportionate amounts. Furthermore if the presence of all three components in similar concentration is required, it is difficult to explain the enormous catalyses produced by cytochrome *c* in the succinic and lactic systems.

It is interesting to calculate how many times per minute each molecule of cytochrome is reduced and oxidised by the lactic dehydrogenase-indophenol oxidase system. Under optimum conditions[1], the oxygen uptake with 1 ml. of 1×10^{-4} *M* cytochrome *c* is 200 μl. per 10 min. or 20 μl. per min. Since a millimol of Fe is equivalent to 5600 μl. of oxygen

$$1 \text{ ml. of } 10^{-4}M \text{ cytochrome} = \frac{5{\cdot}6 \times 10^{3} \times 10^{-1}}{10^{3}} \mu\text{l. } O_2,$$

$$\text{"Wechselzahl"} = \frac{20}{0{\cdot}56} = 35{\cdot}7/\text{min.}$$

Warburg [1934] calculated the number to be 4000 in the intact yeast cell or roughly 100 times as large as in the reconstructed system. This difference is hardly surprising and probably finds its explanation in the fact that the spatial configuration of intact cells cannot be duplicated *in vitro*.

The yellow pigment of bottom yeast has high catalytic activity with respect to the hexosemonophosphate, hexosediphosphate, glucose and malate dehydrogenases. Since the first two systems are quantitatively important in animal and yeast cells, the rôle of the yellow pigment in respiration should be very considerable, provided that the same catalyses occur in the cell. Part II of this series shows that the Q_{O_2} of baker's yeast in presence of hexosemonophosphate or hexosediphosphate is of the same order of magnitude as the maximum Q_{O_2} in presence of glucose or lactate. Hence if the "Wechselzahl" of the yellow pigment is too small to account for more than 0·5 % of the respiration of baker's yeast, it must similarly be too small to account for the oxidation of the very substrates with which yellow pigment is presumed to deal.

A point of great importance which remains unknown is whether the yellow pigments of animal cells have similar catalytic properties to those of the bottom yeast pigment. The alternate oxidation and reduction of yellow pigment in animal cells has not yet been demonstrated. It is therefore premature to speculate about the rôle of the yellow pigment in animal cells.

Flavin is practically completely inactive with respect to all systems except

[1] By optimum conditions are implied excess of lactic dehydrogenase and indophenol oxidase, excess of coenzyme and pure oxygen instead of air.

the malic. The theory that the vitamin action of flavin is associated with oxidation-reduction properties receives little experimental support.

Glutathione presents an entirely different problem from that of the other natural carriers. When yellow pigment, flavin and cytochrome are inactive, it means that the reduction of these carriers is too slow. But in the case of glutathione and systems like the hexosemonophosphate, hexosediphosphate and glucose, the limiting factor is not the reduction process but rather the oxidation process. Where glutathione plays a rôle in respiration, there must be some substance or enzyme system which can catalyse the reaction of reduced glutathione with molecular oxygen. It remains for future research to ascertain the mechanism which the cell possesses for the oxidation of glutathione.

Summary.

1. The ability of cytochrome *c*, glutathione, flavin and yellow pigment to catalyse the reaction of 11 dehydrogenase or oxidase systems with molecular oxygen has been tested.

Cytochrome *c* has catalytic activity only with the succinoxidase of animal tissues and the lactic dehydrogenase of yeast. The "Wechselzahl" of cytochrome *c* when being reduced and oxidised at maximum velocity is 35 per min.

Glutathione, although rapidly reduced by the glucose and hexosemonophosphate dehydrogenases, has little effect on the oxygen uptake. The limiting factor is the rate of autoxidation. Glutathione increases the reaction rate of the hexosediphosphate system with molecular oxygen.

Yellow pigment shows very high catalytic activity towards the glucose, hexosemonophosphate, malate and hexosediphosphate systems.

Flavin is inactive towards all systems except the malic dehydrogenase.

2. New methods for preparing the lactic, succinic, α-glycerophosphoric, glucose and malic dehydrogenases are described. The utility of a steel ball mill for the extraction of enzymes from yeast is shown.

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References.

Adler and Euler (1934). *Z. physiol. Chem.* **226**, 195.
—— —— (1935). *Z. physiol. Chem.* **232**, 6.
Banga, Szent-Györgyi and Vargha (1932). *Z. physiol. Chem.* **210**, 228.
Barnard and Hewlett (1911). *Proc. Roy. Soc. Lond.* B 84, 57.
Bierich, Lang and Rosenbohm (1934). *Z. physiol. Chem.* **223**, 180.
Borsook and Schott (1931). *J. Biol. Chem.* **92**, 535.
Dixon (1927). *Biochem. J.* **21**, 1211.
—— (1934). Manometric methods. (Cambridge.)
—— and Kodama (1926). *Biochem. J.* **20**, 1104.
Euler and Adler (1934). *Z. physiol. Chem.* **226**, 211.
—— —— and Schlötzer (1934). *Z. physiol. Chem.* **226**, 87.
Goszy and Szent-Györgyi (1934). *Z. physiol. Chem.* **224**, 1.
György, Kuhn and Wagner-Jauregg (1934). *Z. physiol. Chem.* **223**, 21.
Green (1933). *Biochem. J.* **27**, 678.
—— (1934). *Proc. Roy. Soc. Lond.* B **114**, 423.
—— and Dixon (1934). *Biochem. J.* **28**, 237.
—— Stickland and Tarr (1934). *Biochem. J.* **28**, 1812.

Haas (1934). *Naturwiss.* **22**, 207.
Harrison (1931). *Biochem. J.* **25**, 1016.
—— (1933). *Biochem. J.* **27**, 382.
Hopkins (1929). *J. Biol. Chem.* **84**, 269.
—— and Elliot (1931). *Proc. Roy. Soc. Lond.* B **109**, 58.
Keilin (1925). *Proc. Roy. Soc. Lond.* B **98**, 312.
—— (1929). *Proc. Roy. Soc. Lond.* B **104**, 206.
—— (1930). *Proc. Roy. Soc. Lond.* B **106**, 418.
Kuhn, György and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 1034.
—— Wagner-Jauregg and Kaltschmitt (1934). *Ber. deutsch. chem. Ges.* **67**, 1452.
—— and Moruzzi (1934). *Ber. deutsch. chem. Ges.* **67**, 1220.
Krebs (1933). *Z. physiol. Chem.* **217**, 191.
—— (1935). *Biochem. J.* **29**, 1620.
Lehmann (1929). *Skand. Arch. Physiol.* **58**, 173.
Mann (1932). *Biochem. J.* **26**, 785.
Meldrum and Dixon (1930). *Biochem. J.* **24**, 472.
—— and Tarr (1935). *Biochem. J.* **29**, 108.
Myrbäck (1933). *Ergebn. Enzymforsch.* **2**, 139.
Pirie (1931). *Biochem. J.* **25**, 614.
Stern (1934). *Biochem. J.* **28**, 949.
Stickland (1929). *Biochem. J.* **23**, 1187.
Wagner-Jauregg, Möller and Rauen (1935). *Z. physiol. Chem.* **231**, 55.
—— Rauen and Möller (1934, 1). *Z. physiol. Chem.* **224**, 67.
—— —— —— (1934, 2). *Z. physiol. Chem.* **228**, 273.
—— —— —— (1934, 3). *Z. physiol. Chem.* **225**, 145.
—— and Ruska (1933). *Ber. deutsch. chem. Ges.* **66**, 1298.
Warburg (1934). *Naturwiss.* **26**, 441.
—— and Christian (1932, 1). *Naturwiss.* **20**, 688.
—— —— (1932, 2). *Biochem. Z.* **254**, 438.
—— —— (1933, 1). *Biochem. Z.* **257**, 492.
—— —— (1933, 2). *Biochem. Z.* **266**, 377.
—— Negelein and Haas (1933). *Biochem. Z.* **266**, 1.