THE EQUILIBRIUM BETWEEN CYTOCHROME OXIDASE AND CARBON MONOXIDE

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Hemoglobin occurs in the bloods of all vertebrates except a few fishes. In invertebrates, however, it appears only sporadically, cropping up in isolated species whose closest relatives often lack this pigment. Indeed here it is distributed so haphazardly as for a time to have discouraged the hope that one might ever discover lines of biochemical relationship among animals comparable with their lines of anatomical relationship. This situation was not improved by the discovery of hemoglobins in Protozoa (Sato and Tamiya, 1937; Keilin and Ryley, 1953); in the root nodules of leguminous plants (Keilin and Wang, 1945); and in a number of molds (Keilin, 1953; Keilin and Tissières, 1953).

With the realization, however, that hemoglobin is closely related chemically to the heme enzymes of cellular respiration—cytochrome oxidase, the cytochromes, catalase, and the peroxidases—this situation became more understandable. Such enzymes occur in all aerobic cells, and it seems probable that in the course of evolution hemoglobins have repeatedly been derived from them (Barcroft, 1928, chapter 4; Anson and Mirsky, 1930, p. 525).[1]

In an attempt to define this development more closely it has been suggested that the hemoglobins evolved in three stages, involving three fundamental properties (Wald, 1952). The stages were: (1) Heme enzymes, among which one thinks first of cytochrome oxidase, the only member of this class that unites directly with oxygen and carbon monoxide. (2) Storage hemoglobins, concerned primarily with *holding* oxygen; *i.e.*, with increasing the oxygen *capacity* of the cells and tissues. (3) Circulatory hemoglobins, concerned primarily with *transporting* oxygen. The properties of hemoglobin that alter progressively in this development are: (1) The affinity for oxygen decreases. (2) The shape of the oxygen equilibrium curve—percentage oxygenation *vs.* oxygen pressure—changes from a rectangular hyperbola to a more and more sharply inflected S.

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[1] Compare Anson and Mirsky (1930, p. 525): "Hemoglobin, it now appears, may no longer be regarded as the pre-eminent heme pigment, nor is its haphazard distribution now so mysterious. Hemoglobin is merely an occasional specialized derivative of an iron substance, heme, which is much more widely distributed."

(3) The affinity for oxygen declines more and more sharply with rise in acidity: the Bohr effect.

This development is cumulative; vertebrates in general possess all its stages: cytochrome oxidase in all the cells; the typical storage hemoglobin, myoglobin, in the muscles; a circulatory hemoglobin in the blood. Indeed a single organ such as the beef heart, from which we derived our cytochrome oxidase, displays all these stages.

Muscle and blood hemoglobins are already known to be related in their properties as here described. Muscle hemoglobin has a relatively high affinity for oxygen, a hyperbolic oxygen equilibrium curve, and a very small Bohr effect; blood hemoglobin a much lower oxygen affinity, an S-shaped equilibrium curve, and a relatively large Bohr effect.

Of cytochrome oxidase in this regard one knows only that it possesses a relatively high affinity for oxygen. The evolution argument reviewed above implies that it should also possess an approximately hyperbolic equilibrium function, and little if any Bohr effect. The object of the present investigation is to inquire into these properties.

The reaction of oxygen with cytochrome oxidase, however, is quite different from that with hemoglobin. The former is a true oxidation, changing the iron of cytochrome oxidase from the ferrous to the ferric condition; whereas the reaction with hemoglobin is only an "oxygenation" which leaves the iron ferrous as before. Cytochrome oxidase does not enter into an equilibrium with oxygen, but is oxidized by it.

For this reason we studied instead the reaction between cytochrome oxidase and carbon monoxide. This is a true equilibrium, strictly analogous with that between carbon monoxide or oxygen and hemoglobin. In all hemoglobins so far examined, though the affinities for oxygen and carbon monoxide may differ widely, the shape of the equilibrium function and the vigor of the Bohr effect appear to be the same with both gases (*cf.* Barcroft, 1928 pp. 148–160). For these two properties therefore the reaction with carbon monoxide provides a reliable analogy to that with oxygen.

In a sense therefore, in these experiments, we examine cytochrome oxidase as though it were a hemoglobin. Nevertheless our observations bear also upon its general properties, as an enzyme and a heme protein.

Methods

Preparation.—The beef heart preparation of Ball and Cooper (1949) was used as the source of cytochrome oxidase. The ammonium sulfate precipitate was suspended in distilled water, and centrifuged for 1 hour at 5°C., at 25,000 R.P.M. (41,200 *g*) in the Spinco preparative ultracentrifuge. The clear, colorless supernatant was discarded. The brown pellet was transferred to a drying tube, frozen in a bath of dry ice in acetone, and lyophilized. The resulting powder was extracted twice with pe-

troleum ether. The last traces of petroleum ether were removed under vacuum, and the powder stored at −20°C. until required.

To dissolve the cytochrome components, 0.120 gm. of the dried powder was ground in a Potter-Elvehjem homogenizer with 4 ml. of 2 per cent sodium desoxycholate (L. Light and Co.) in M/20 glycylglycine buffer at pH 8.3. The resulting suspension was centrifuged for an hour at 25,000 R.P.M. in the Spinco ultracentrifuge. The clear brown supernatant was decanted and used in our experiments. Its pH was about 7.4.

Carbon Monoxide Equilibrium.—The tonometer was similar to that described by Allen, Guthe, and Wyman (1950), but adapted to fit into the Beckman spectrophotometer. The opening for injection of liquids and gases had been moved to the side of the equilibration chamber. The optical path was 1 cm.

The sodium desoxycholate solution of cytochrome components (3 ml.) was pipetted into the tonometer, and the latter sealed with a rubber vaccine cap. Air was removed by successive evacuations through the stopcock and by filling with nitrogen. Evacuation was by water aspirator, with the tonometer immersed in cracked ice to prevent foaming. The solution in the tonometer was then equilibrated by rotation in a water bath for 15 minutes.

During this time a few milliliters of 2 per cent desoxycholate solution in glycylglycine buffer was washed with nitrogen to remove oxygen.

To reduce the cytochrome system, about 10 mg. of sodium dithionite ($Na_2S_2O_4$) was placed in a dry syringe, and 1 ml. of the sodium desoxycholate solution under nitrogen drawn into it. 0.5 ml. of the resulting solution was injected immediately into the tonometer through the vaccine cap. This brings the pH of the cytochrome preparation to about 7 unless alkali is added to the buffered desoxycholate used to dissolve the dithionite. The tonometer was again evacuated and rinsed with nitrogen, usually three times. Finally it was equilibrated for 20 minutes under vacuum.

Nitrogen and carbon monoxide were mixed at constant volume, in proportions determined by measuring the pressure with a mercury manometer. The mixtures were stored in a bottle under positive pressure. The bottle had five outlets, sealed with rubber vaccine caps, from which gas could be taken with a calibrated hypodermic syringe. The syringe was rinsed three times with the gas mixture, then filled and a measured amount of the gas injected into the tonometer through the rubber cap. The tonometer was then rotated for 20 minutes in a water bath at a temperature of 10° ± 0.1°C. Tests showed this period to be sufficient for attaining equilibrium. Further details of these procedures have been described by Allen *et al.* (1950) and by Riggs (1951–52).

After equilibration with each pressure of carbon monoxide, the extinction of the preparation was measured at 445 and 650 mμ. These measurements were made in a cold room kept at 10° ± 2°C. After such determinations had been completed at five different pressures of CO, the tonometer was filled with pure CO and reequilibrated for 20 minutes. Then the entire absorption spectrum was measured from 440 to 650 mμ.

Finally the tonometer was allowed to warm to room temperature and the pH of the preparation determined with a Beckman pH meter, immediately on removal from the tonometer.

Enzyme Activity.—This was measured by the method of Potter (*cf.* Umbreit *et al.*,

1949). The preparation, to which cytochrome *c* in phosphate buffer and dilute aluminum chloride had been added, was placed in the main chamber of a Warburg manometer, with sodium ascorbate in the side compartment. After thermal equilibrium was established, the ascorbate was tipped into the main chamber, and the oxygen uptake measured. The solution was then stored overnight in the refrigerator, and its absorption spectrum measured in the oxidized state, reduced, and combined with CO.

Observations

Absorption Spectra.—The absorption spectra of all our preparations were essentially identical. Fig. 1 shows a representative series, all at equivalent concentration: the preparation in air (oxidized), reduced with dithionite, and treated with carbon monoxide. Below 480 mμ the extinctions rise rapidly, and the continuation of the spectra in this region is shown on a more compressed scale in Fig. 2. The difference spectrum of the carbon monoxide complex—the difference in absorption spectrum between it and the reduced preparation—is shown in Fig. 3.

These spectra are very similar to those published by Ball, Strittmatter, and Cooper (1951) on a similar preparation. Since our experiments were completed (1952), such preparations have been resolved further, so as to yield essentially mixtures of cytochrome oxidase and cytochrome *a* (Smith and Stotz, 1954; Smith, 1955*a*,*b*; Wainio, 1955). Our preparation contained in addition cytochrome *b* and some *c*; but there is no reason to believe that this should have influenced the results of our measurements.

The oxidized preparation displays little more than absorption rising continuously into the ultraviolet, owing probably in large part to light scattering. Superimposed on this is a small absorption peak at about 525 mμ and a small inflection at about 590 mμ. On reduction the bands of the cytochromes appear: *a* at about 605 mμ, *b* and *c* at 552 to 562, and a band at 525 mμ attributable to all three cytochromes.

On exposure to CO, small changes occur in most parts of the spectrum. These appear to involve specifically cytochrome oxidase. The only contaminants that would react with CO are blood and muscle hemoglobins; and we believe these to be virtually absent from our preparations for the following reasons: (*a*) washings of our preparations with distilled water are colorless; (*b*) the oxidized preparations show no trace of the oxyhemoglobin peaks at 540 and 570 mμ; and (*c*) the preparations exposed to CO show no sign of the shift of absorption toward the red below 650 mμ that would accompany the formation of CO-hemoglobin (*cf.* Horecker, 1943). On the contrary the CO-complex formed with our preparations displays the lowest absorption at these wave lengths (Fig. 1).

The difference spectrum shown in Fig. 3 therefore appears to represent cytochrome oxidase. It is almost identical with that obtained by Ball, Strittmatter,

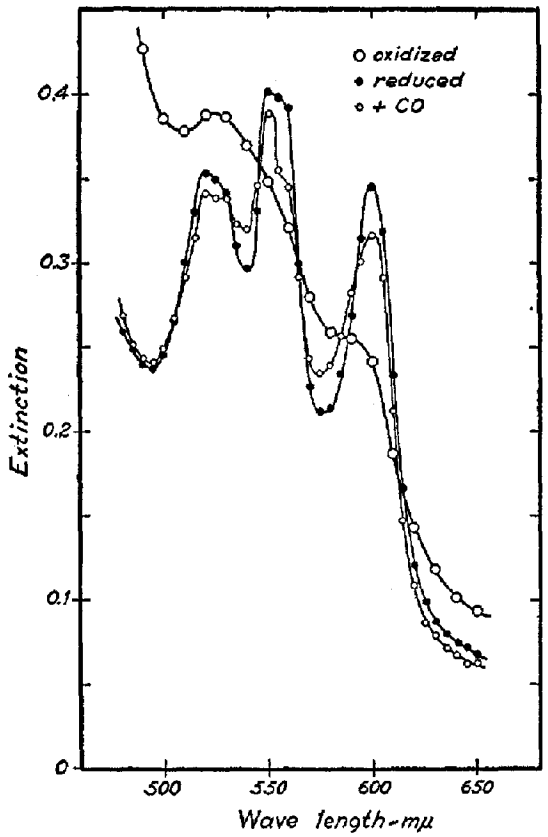

FIG. 1. Absorption spectra of a preparation of cytochrome system components of beef heart in sodium desoxycholate solution: oxidized in air; reduced with dithionite; and saturated with carbon monoxide. pH 7.60. The absorption bands in the reduced preparation display the presence of cytochromes *a* and *b*, and a small amount of *c*. The changes in spectrum caused by carbon monoxide appear to be due entirely to cytochrome oxidase.

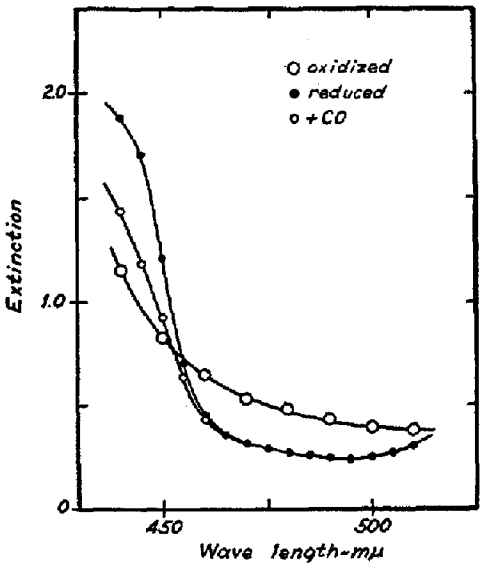

FIG. 2. Extension of the spectra of Fig. 1 to lower wavelengths, plotted on a compressed scale of extinctions.

and Cooper (1951) from a similar preparation, and by Chance and Williams (1955) for the cytochrome oxidase component of suspension of rat liver mitochondria. It is this difference spectrum that guided our choice of the wave lengths used to measure the carbon monoxide equilibrium: 445 and 650 mμ. Our calculations are based on the changes at 445 mμ; while the measurements

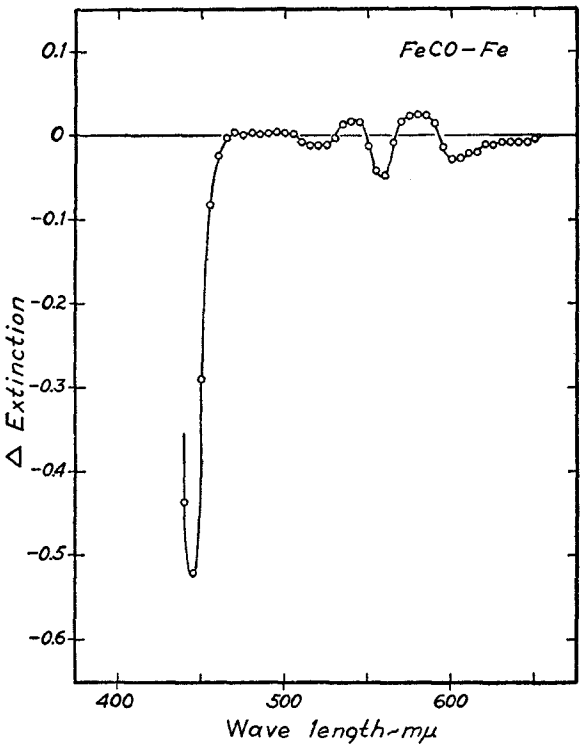

FIG. 3. Difference spectrum of the carbon monoxide complex of beef heart cytochrome oxidase. To obtain this the absorption spectrum of the reduced preparation was subtracted from that of the preparation saturated with carbon monoxide (Figs. 1 and 2).

at 650 mμ, where no changes are expected from cytochrome oxidase, were used to control changes in light scattering and other extraneous disturbances that might affect the course of the measurements.

Carbon Monoxide Equilibrium.—Presumably in cytochrome oxidase as in hemoglobin, one molecule of CO combines with one atom of Fe. We do not yet know how many Fe-porphyrin units one molecule of cytochrome oxidase contains; or if more than one, to what degree they interact with one another. The

general equation for the combination of cytochrome oxidase with CO may be written:

$$Fe_n + n\ CO = (FeCO)_n$$

in which n is an empirical constant, not necessarily an integer, which represents the degree to which the Fe-porphyrin units of cytochrome oxidase interact in their combination with carbon monoxide. If the enzyme contains only one such unit per molecule, or if the units combine with CO independently of one another, $n = 1$.

The corresponding mass action equation can be written:

$$K = \frac{[(FeCO)_n]}{[Fe_n]pCO^n}$$

in which pCO is the partial pressure of CO in the gas phase, and K, the equilibrium constant, measures the affinity of cytochrome oxidase for carbon monoxide.

When the enzyme is half-saturated with CO, pCO may be written as p_{50}; and since $(FeCO)_n = Fe_n$,

$$p_{50}^n = 1/K$$

The constant p_{50} is therefore a useful inverse measure of the affinity, and a means of evaluating K.

For some purposes these relationships are expressed more conveniently in Hill's equation:

$$\frac{y}{100} = \frac{Kp^n}{1 + Kp^n}$$

in which y is the percentage saturation, p the CO pressure, and K and n have their earlier meanings. When $n = 1$, this is the equation of a rectangular hyperbola; when n is greater than 1, the curve is S-shaped.

To evaluate n it is most convenient to throw this and the preceding equation into logarithmic form:

$$\log K = -n \log p_{50}$$

$$n \log \frac{p}{p_{50}} = \log \frac{y}{100 - y}$$

This last is the equation of a straight line of slope n.

The results of five experiments made with two different preparations are summarized in Table I and in Figs. 4 and 5.

In Fig. 4 all the data are plotted in the conventional way, that of the Hill equation. p_{50} in all the experiments lies between 0.16 and 0.18 mm.; the average

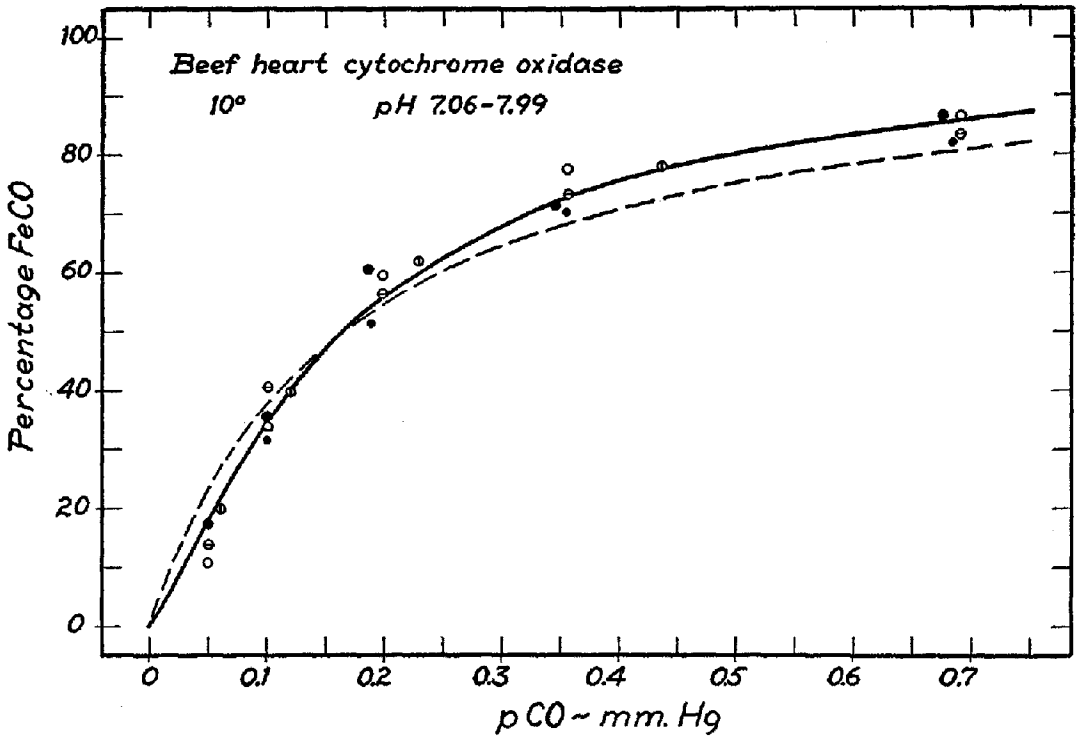

FIG. 4. The equilibrium between beef heart cytochrome oxidase and carbon monoxide: percentage saturation (y) plotted against partial pressure of carbon monoxide (p). Data of five experiments involving two different preparations. The solid line is drawn according to the Hill equation, $y/100 = 9.57\ p^{1.26}/(1 + 9.57\ p^{1.26})$. The broken line shows the corresponding rectangular hyperbola, in which the exponent of the above equation has been changed from 1.26 to 1.

TABLE I

Data of five experiments performed with two different preparations of beef heart cytochrome system components dissolved in 2 per cent sodium desoxycholate, and buffered with glycylglycine. p_{50} is the partial pressure of carbon monoxide at which the cytochrome oxidase is 50 per cent saturated; n is the exponent in the Hill equation. All preparations equilibrated at 9.8° ± 0.1° C.

Preparation	pH	p_{50} (mm. Hg)	n
I	7.06	0.162	1.36
II	7.60	0.178	1.26
	7.84	0.182	1.26
	7.38	0.148	1.27
	7.99	0.155	1.26
	Averages	0.165	1.28

value is 0.165 mm. (Table I). This way of plotting the data suggests the presence of a slight inflection; and this is confirmed by the logarithmic plot of the same data in Fig. 5. The slope of the line in Fig. 5 is $n = 1.26$; the value $n = 1$

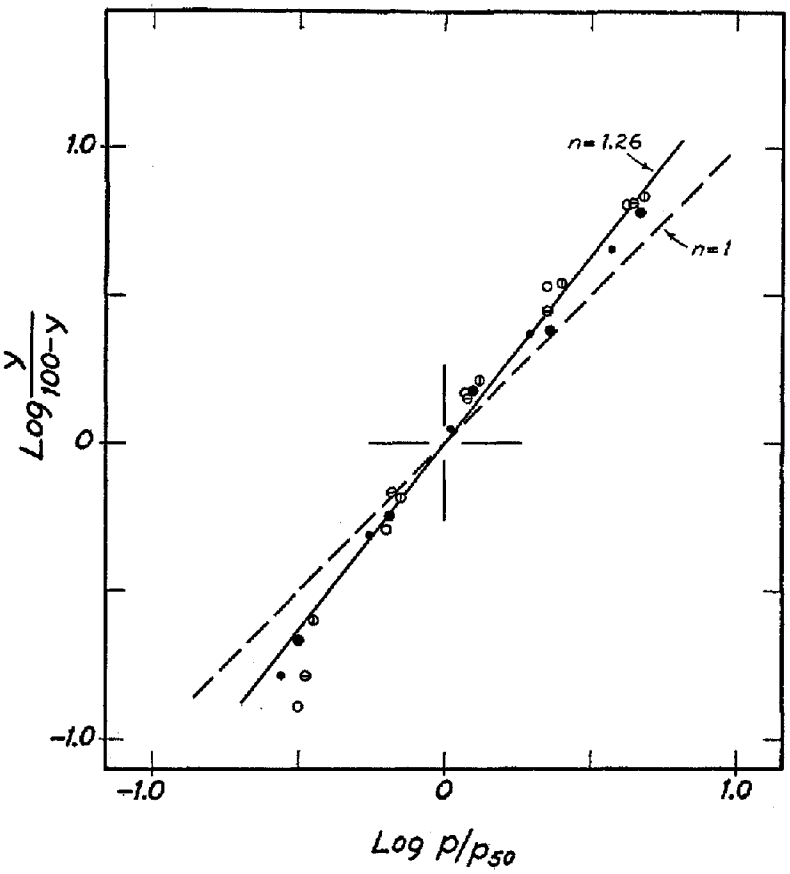

FIG. 5. The data of Fig. 4 replotted in logarithmic form, so as to yield a straight line of slope n. The best fit of these data shows that $n = 1.26$. The line for $n = 1$, implying that the oxygen equilibrium curve is a rectangular hyperbola, clearly is inadequate.

clearly is inadequate. Using $n = 1.26$ and $p_{50} = 0.165$, $K = 9.57$, and the Hill equation for these data is:

$$\frac{y}{100} = \frac{9.57p^{1.26}}{1 + 9.57p^{1.26}}$$

This equation was used to draw the solid curve in Fig. 4. The corresponding rectangular hyperbola ($n = 1$; other constants as before) is drawn in this

figure with a broken line. The rectangular hyperbola fails by very little, yet significantly, to describe the data.

Bohr Effect.—The above measurements were made at pH's varying between 7.06 and 7.99. Over this range the affinity for CO does not change significantly, as shown by the constancy of p_{50} (Table I, Fig. 6). It can be concluded that at least over this range cytochrome oxidase exhibits no Bohr effect.

Enzymatic Activity.—As evidence that our preparations were in good order, we measured the enzyme activity of one of them (II). The procedure was described under "Methods." The uptake of oxygen was linear for about 35 minutes, then fell off. In two Warburg vessels, to one of which twice as much cytochrome oxidase had been added as to the other, the ratio of rates of oxygen uptake was 1.7. The activity of the solution which yielded the difference spectrum of Fig. 3 was 3410 μl. O_2/ml./hr.

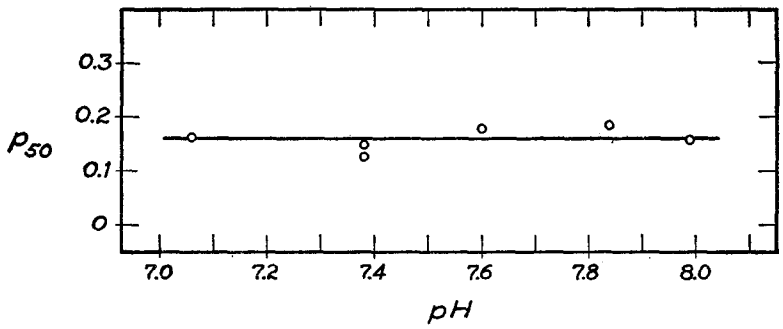

FIG. 6. The variation of p_{50} with pH. The affinity of cytochrome oxidase for carbon monoxide does not change from pH 7 to 8; *i.e.*, there is no Bohr effect in this range.

DISCUSSION

From the point of view of the evolution argument reviewed above, these experiments have come out reasonably well. Among the properties with which the argument was concerned, cytochrome oxidase takes an extreme position in two, and nearly so in the third. In affinity for oxygen it was already known to exceed all the hemoglobins with which it is associated. We can now add that it lacks the Bohr effect, as does no known hemoglobin. In the usual mammalian blood hemoglobin, p_{50} is increased 3 to 4 times by a fall of pH from 8 to 7; in horse muscle hemoglobin it rises about 25 per cent (Theorell, 1934); in beef cytochrome oxidase it remains unchanged. In the third property, the shape of the equilibrium function, cytochrome oxidase has nearly the extreme hyperbolic form, though departing from this measurably.

All such evolutionary arguments are no more than orderly arrangements of data, fitting what would otherwise be unrelated facts into the context of a larger construction. At best one hopes to derive from them some stimulus for

further inquiry, and some measure of prediction of further observations. The present argument can be said to be serving these purposes.

What justification exists for transposing information gained with carbon monoxide to the combination of cytochrome oxidase with oxygen? First, as to hemoglobins: with regard to the properties we have examined—the interaction of Fe-porphyrin groups and the Bohr effect—hemoglobins display an extraordinary identity of behavior toward oxygen and carbon monoxide (Barcroft, 1928, pp. 148–160). One would like to assume that some such parallelism holds also for cytochrome oxidase; but here one must meet the problem that this enzyme reacts quite differently with both gases. As already noted, with carbon monoxide it enters a true equilibrium, whereas with oxygen it undergoes an intrinsically irreversible oxidoreduction. How then compare them?

The point is that in the presence of suitable reducing systems, cytochrome oxidase is continuously reduced, and continuously reoxidized by oxygen. If only a limited amount of reductant is available, the rate of reduction will fall off with its concentration. More usually, however, reductants are present in excess, and in this condition the rate of reduction depends only upon the concentration of oxidized enzyme. The enzyme then enters a steady state or pseudoequilibrium, in which its rate of reduction is equal to its rate of oxidation. For the steady state one can write the equation:

$$K' = \frac{[(FeO_2)_n]}{[Fe]_n pO_2^n}$$

in which Fe represents the Fe-porphyrin groups—one or more—which combine with a single molecule of oxygen; and n represents the degree of interaction among the oxygen-combining centers. That is, such a steady state equation has exactly the same form as the equation for the equilibrium of cytochrome oxidase with carbon monoxide, or of hemoglobin with either gas (*cf.* Warburg, 1927). In the presence of excess reductant, the pseudoequilibrium between cytochrome oxidase and oxygen can be treated just as are these true equilibria.

From this point of view the properties we have considered probably have considerable importance in the physiological functioning of cytochrome oxidase. The business of this enzyme is to make oxygen available for cellular respiration. Its high affinity enables it to take oxygen away from blood and tissue hemoglobins, and to maintain maximal rates of respiration down to very low oxygen pressures. The nearly hyperbolic form of its (pseudo-) equilibrium curve—which we assume, as in the hemoglobins, to be much the same with oxygen as with carbon monoxide—facilitates a high degree of saturation at low oxygen pressures. The absence of Bohr effect—which again as in the hemoglobins we assume to be the same with oxygen as with carbon monoxide—means that in conditions involving oxygen debt, as in anoxia or violent work, as the cell grows more acid, this change does not interfere with its respiration.

In these properties cytochrome oxidase lies at the other extreme from a circulatory hemoglobin. The business of the latter is not only to combine with oxygen in the gills or lungs, but to discharge it in the tissues, a function promoted by just those properties cytochrome oxidase lacks: a low affinity for for oxygen, a strongly inflected S-shaped equilibrium curve, and a large Bohr effect.

The pressures of carbon monoxide at which our preparations were half-saturated—0.16 to 0.18 mm.—seem surprisingly low. In part this is the result of the low temperatures at which these measurements were made. If we can assume as with hemoglobin that p_{50} rises about 3 times for each 10° rise in temperature (*cf.* Barcroft, 1928, p. 174), the p_{50} should be about 3 mm. at 37.5°C. In recent measurements with a beef heart particle system at pH 7.37 and 37.7°C., Ball *et al.* (1951) found the oxidation of two substrates to attain half the maximal rate at about 10 mm. oxygen. This represents the p_{50} of the cytochrome oxidase–oxygen pseudoequilibrium *under these conditions of reduction.*

These measurements make it appear that cytochrome oxidase has a higher affinity for carbon monoxide than for oxygen; yet just the contrary is commonly stated to be true. When intact cells or cytochrome preparations are exposed to mixtures of carbon monoxide and oxygen, and rates of respiration or oxidation are measured, it appears that to inhibit such rates 50 per cent requires that the gases be in the ratio $CO/O_2 = 2.2 - 28$ (Warburg, 1926; *cf.* review of such data by Ball *et al.*, 1951). These values have somewhat the force of p_{50} ratios for the two gases in their combination with cytochrome oxidase, and imply that oxygen has much the greater affinity.

As already remarked, however, though the reaction with carbon monoxide is a simple, reversible addition, yielding a true equilibrium, the reaction with oxygen is an intrinsically irreversible oxidation, yielding at most a pseudo-equilibrium or steady state in the presence of excess reductant. (In the latter instance such a term as "affinity" has only an arbitrary meaning; the p_{50} depends directly upon the velocity of reduction.) This distinction lends oxygen a great advantage when both gases are placed in competition. It is probably true, as our measurements show, that carbon monoxide, when not competing with oxygen, has a higher affinity for cytochrome oxidase than had been supposed; yet for the reason given it is only a feeble respiratory poison.

The departure of the carbon monoxide equilibrium curve from a hyperbola is so small as to have little physiological effect in itself; yet it carries the important implication that since the Fe-porphyrin groups of cytochrome oxidase interact to a degree, this enzyme must contain *more than one Fe-porphyrin unit per molecule.*

The interaction raises a further consideration. The reaction of cytochrome oxidase with oxygen involves both combination and oxidation, and so could

include two distinct types of interaction: (1) the combination of the enzyme with a first molecule of oxygen could facilitate the binding of a second molecule of oxygen; and (2) Fe-porphyrin units could cooperate in reducing oxygen.

Much has been made recently of the thought that the chain of respiratory processes passes from systems concerned with two-electron transport (DPN and flavine enzymes) to others concerned with one-electron transport (metalloenzymes, including those of the cytochrome system). It should not be forgotten, however, that at the end of the chain lies the diatomic molecule, O_2, which requires *two* electrons for its reduction. The passage of one electron to oxygen yields the superoxide ion, O_2^-, or in water the radical HO_2, both highly unstable in the free state; whereas two electrons form the stable peroxide ion, $O_2^=$, or in water H_2O_2.

If the Fe-porphyrin groups of cytochrome oxidase united with oxygen in the same proportion as in the hemoglobins ($Fe^{++}:O_2$), only one electron could be transmitted at a time. One would have to assume a reduction of oxygen in two stages, passing over the unstable intermediate O_2^-. Such a sequence of reactions presents obvious kinetic and thermodynamic difficulties.[2]

It is intrinsically more probable that in cytochrome oxidase *two* Fe-porphyrin units combine with one molecule of oxygen in the manner Fe^{++}–O_2–Fe^{++}. In this case two electrons could pass almost simultaneously from iron to oxygen, yielding two ferric ions and releasing the oxygen as hydrogen peroxide. Such an arrangement should bring cytochrome oxidase into close relationship with hemocyanin, in which copper unites with oxygen in the proportion 2 $Cu^+:O_2$, with the resultant oxidation of one or both cuprous ions (Schulman and Wald, 1951; Klotz and Klotz, 1955); and with hemerythrin, in which iron appears to combine with oxygen in the proportion 2 $Fe^{++}:O_2$, with the consequent oxidation of both ferrous ions (Klotz and Klotz, 1955).

On the other hand it is unlikely that carbon monoxide can form a similar bridge between two iron atoms. In cytochrome oxidase iron probably combines with carbon monoxide in the same proportion as in the hemoglobins, Fe^{++}:CO. It is possible therefore that in this enzyme two Fe-porphyrin units that facilitate each other in combining with *two* molecules of CO, combine with *one* molecule of oxygen. In that case the equilibrium curve which we found slightly inflected with carbon monoxide ($n = 1.26$) might become a rectangular hyperbola with oxygen ($n = 1$).[3] Our observations, therefore, need not be in conflict

[2] This discussion owes much to Michaelis (1948), who remarked upon the almost unique peculiarity of hemoglobin in combining ferrous ions with oxygen without oxidation, and went on to say, "One may wonder whether the function of—globin in hemoglobin is to fix the position of the heme molecules—so that an oxygen molecule attached to one of them can never form a bridge to another."

[3] It is interesting that reduced alkali-denatured hemoglobin ("globin-hemochromogen") yields a hyperbolic equilibrium function with carbon monoxide. (Anson and

with the report of Ludwig and Kuby (1955) that the percentage oxidation of cytochrome oxidase rises hyperbolically with oxygen concentration in living yeast.

These considerations yield some insight into the possible intramolecular mechanism of the evolution of hemoglobins from cytochrome oxidase. Let us assume, pending further examination, that in cytochrome oxidase two Fe-porphyrin units combine with one molecule of oxygen in the arrangement Fe^{++}–O_2–Fe^{++}, so facilitating the oxidoreduction that is the principal business of this enzyme. The first step in forming a hemoglobin would involve separating the Fe-porphyrin units, so that each combines independently with oxygen (Fe^{++}–O_2) (*cf.* Michaelis, 1948.). The iron would then tend to remain ferrous; oxidation would give way to oxygenation. A further consequence of separating the hemes would be the almost complete loss of interaction; the equilibrium curve would be close to hyperbolic, n nearly equal to 1. These are the properties of the typical storage hemoglobins of muscle and most invertebrate bloods. Finally, a new mechanism of interaction among the hemes is evolved, which, while still preventing the formation of an oxygen bridge between two iron atoms, mutually facilitates the combination with oxygen. This has given us the typical circulatory hemoglobins of vertebrate blood, with strongly inflected oxygen equilibrium curves (n = 2 to 3). The mode of attachment of heme groups to globin holds them apart sufficiently so that an oxygen molecule can unite with only one. The interaction is transmitted through the protein, and apparently involves sulfhydryl groups (Riggs, 1952–53; Riggs and Wolbach, 1955–56).

It is necessary only to denature oxyhemoglobin to have it oxidize rapidly to methemoglobin (Anson and Mirsky, 1925). Possibly the disorganization of the globin structure permits Fe-porphyrin units to come together, and so co-operate in passing electrons to oxygen, as we suppose happens normally in cytochrome oxidase.

SUMMARY

An evolution argument which attempted to trace the development of hemoglobins from such respiratory pigments as cytochrome oxidase presupposed that the latter possesses, in addition to its high affinity for oxygen, an approxi-

Mirsky, 1925). The denaturation of globin abolishes the interaction among the hemes, so that each apparently combines independently with CO. "Haemoglobin is not merely an organic compound perhaps a bit more complicated than the rest. It is a biological molecule. It has structure, just as an organism has structure. It has organisation, just as an organism has organisation. Destroy that structure. What is left has the same chemical composition as at the start. But all the biologically valuable qualities have disappeared. Haemochromogen, despite its iron and its pyrrol and its protein, is dead and useless" (Anson and Mirsky, 1925).

mately hyperbolic equilibrium function, and little if any Bohr effect (decline in affinity for oxygen with rise in acidity). Since cytochrome oxidase, unlike hemoglobin, is irreversibly oxidized by oxygen, the present experiments examine its combination with carbon monoxide, with which, like hemoglobin, it yields a true equilibrium. In all known hemoglobins the form of the equilibrium function and the vigor of the Bohr effect are similar with carbon monoxide and with oxygen, so that observations involving the former gas are relevant to the relations of the latter.

The equilibrium function of cytochrome oxidase with carbon monoxide—percentage saturation *vs.* partial pressure of CO—is slightly inflected (in the Hill equation $n = 1.26$; for a hyperbola, $n = 1$). No Bohr effect is present in the range of pH 7–8. The pressure of carbon monoxide at which half-saturation occurs (p_{50}) is about 0.17 mm. at 10–13°C. The affinity for carbon monoxide is therefore higher than commonly supposed.

These properties are consistent with the evolution argument. They are important also for the physiological functioning of cytochrome oxidase, the nearly hyperbolic equilibrium function facilitating a high degree of saturation, and the lack of Bohr effect making this enzyme impervious to hyperacidity.

The slight inflection of the equilibrium function shows that the Fe-porphyrin units of cytochrome oxidase interact to a degree, hence that the enzyme must contain more than one such unit per molecule. It is suggested that in cytochrome oxidase two Fe-porphyrin groups may unite with one oxygen in the manner Fe^{++}–O_2–Fe^{++}; and that the evolution of hemoglobins proceeded over a first stage in which the hemes were separated so that each combines with only one molecule of oxygen, so tending to remain reduced; to a further stage in which the separated hemes interact through the protein to facilitate one another in combining with oxygen.

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