sumably depend upon how far the specific configuration is determined by amino-acid sequence and how far by the influence of other spacially adjacent sections of the molecule.

SUMMARY

1. The effect of a wide variety of hydrolytic conditions on the immunological activity of rabbit anti-ovalbumin has been examined.

2. Papain-hydrocyanic acid hydrolysis produces a quarter molecule unable to flocculate with the antigen, but able to inhibit flocculation of the complete system.

3. This inhibitory fragment appeared to have arisen from the terminal quarter of the molecule.

4. Attempts to reduce the size of the inhibitor

further without destroying its activity were only partially successful.

5. A study of the interactions of antibody, inhibitor and antigen showed that complete inhibition of flocculation could be produced. Inhibition or flocculation could be reversed and the conclusion was reached that inhibitor was probably competing with antibody for the same combining centres of the antigen.

6. There was no evidence to suggest that the inhibitor was carried down by the specific precipitate when flocculation occurred in its presence.

I wish to thank Prof. J. R. Marrack for his valuable criticism and Prof. A. C. Chibnall for his interest in this work. The receipt of a personal grant from the Medical Research Council is gratefully acknowledged.

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The Components of the Dihydrocozymase Oxidase System

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(Received 10 November 1949)

It has been known for many years that cozymase is necessary for the oxidation of a number of intermediary metabolites by tissues. The cozymase (Co I) is reduced by the metabolite, activated by a specific dehydrogenase, to dihydrocozymase (Co I H₂). There are two well known ways whereby the Co I H₂ may be oxidized to reform Co I, viz. (1), by the anaerobic reaction with a substrate (S): $S + \text{Co I H}_2 \rightarrow \text{Co I} + \text{SH}_2$, which requires a single enzyme, SH_2 dehydrogenase; and (2), by the aerobic reaction

which requires a complex of enzymes, which we may call the Co I H₂ oxidase system. The present investi-

gation, which is primarily concerned with the elucidation of the components of this Co I H₂ oxidase system, led to a study of a less well known third method for the oxidation of Co I H₂, viz. its anaerobic oxidation by fumarate: $\text{Co I H}_2 + \text{fumarate} \rightarrow \text{Co I} + \text{succinate}$. This reaction was demonstrated indirectly by Dewan & Green (1937*a*), who found an oxido-reduction linking β -hydroxybutyrate, which is a typical cozymase-requiring dehydrogenase, and succinic dehydrogenase, which does not require cozymase. Its mechanism has not hitherto been studied. It is shown below that this is a slow reaction, and it is somewhat doubtful whether it is of great quantitative importance *in vivo*.

While it is widely, although not universally, accepted that the Co I H_2 oxidase system includes diaphorase or Co I H_2 dehydrogenase (Dewan & Green, 1937*b*, 1938; Adler, Euler & Hellström, 1938; Straub, 1939) and cytochrome oxidase, there is no agreement concerning the components of the cytochrome system involved. This uncertainty regarding the reaction between diaphorase and cytochrome oxidase constitutes, in fact, a serious gap in our knowledge of intracellular respiration. Dewan & Green (1937*b*, 1938) believed that cytochromes *a* and *b*, but definitely not *c*, were required. Lockhart & Potter (1941), on the other hand, obtained good evidence for the participation of *c*. Okuniki & Yakushiji (1940) thought that *b* and *c* were definitely required, while the role of *a* was uncertain. The present investigation shows that cytochromes *c*, *a* and *a*₃ (but not cytochrome *b*) and the BAL-sensitive factor previously found as a component of the succinic oxidase system (Slater, 1948, 1949*a*) are components of the Co I H_2 oxidase system. Some of the findings of the present investigation have been reported briefly (Slater, 1949*b*).

METHODS

Enzyme preparations

Heart-muscle preparation was prepared essentially by the method previously described (Keilin & Hartree, 1947; Slater, 1949*c*), with the exception that the precipitate collected by centrifugation after bringing the pH to 5.7 was washed by rubbing with ice-cold water and recentrifuging. This washing did not reduce the Co I H_2 oxidase activity. The precipitate was then suspended in an equal volume of 0.25*M*-phosphate buffer, pH 7.3 (cf. 0.1*M* previously used). The solution was kept at 4° and used within 4 days of preparation. The Co I H_2 oxidase system in this preparation is much less stable than the succinic oxidase system; after 6 days at 4°, the Co I H_2 oxidase activity was only one-half of its initial value, while the succinic oxidase system was only slightly affected.

Kidney preparation was prepared in the same way as previously (Slater, 1949*c*), except that 0.9% NaCl was used for washing instead of water (cf. Schneider, Claude & Hogeboom, 1948).

Cytochrome c was the product with 0.34% Fe, prepared by the method of Keilin & Hartree (1945).

Diaphorase was prepared from heart muscle by a method similar to that of Straub (1939).

Lactic dehydrogenase was a semi-purified preparation. Straub's (1940*a*) method was followed up to the first precipitation with $(NH_4)_2SO_4$. The precipitate thus obtained was dissolved in water and fractionated with $(NH_4)_2SO_4$, the fraction precipitating between 0.3 and 0.6 saturation being collected and dissolved in 0.3 saturated $(NH_4)_2SO_4$.

Liver extract. This was used as the source of glutamic dehydrogenase. Sheep or horse liver was cut into pieces with scissors and treated with 5 vol. of ice-cold 0.02*M*-phosphate buffer, pH 7.3, in a Waring blender for 2 min. The mixture was centrifuged at 2000 rev./min. (1250 *g*) for 20 min. in an International centrifuge (size 1) with 250 ml. cups and

the cloudy supernatant centrifuged at 12,000 rev./min. (13,000 *g*) in a Servall angle centrifuge (type SS1) for 30 min. The supernatant was dialysed against changes of distilled water for 18 hr. at 4°. A small precipitate was removed by centrifuging in the Servall centrifuge for 30 min. at 12,000 rev./min. This extract was used on the day of preparation.

Cozymase

Preparation. Cozymase, prepared from baker's yeast by an unpublished modification by Ochoa of Williamson & Green's (1940) method, was 33–34% pure. (This purity is not as high as is usually obtained by Ochoa's (Dr Ochoa, personal communication) method, probably because of the strain of yeast used.) The coenzyme π content, kindly determined by Dr C. Liébecq, by its catalytic activity in the oxidation of glucose-6-phosphate (in the presence of its dehydrogenase) by $K_3Fe(CN)_6$, was less than 0.2%.

Determination of purity. Cozymase preparation (10 mg.) was dissolved in 2 ml. of 1.3% (w/v) $NaHCO_3$ in a manometric flask containing 5 mg. of $Na_2S_2O_4$ in a dangling tube. The flask was attached to a manometer and the gas phase made 95% N_2 –5% CO_2 (v/v). After tipping the dangling tube into the solution, the reaction was allowed to proceed at room temperature until the evolution of CO_2 ceased (3–5 hr.). This method of reduction is essentially that of Ohlmeyer (1938). The flask was then removed from the manometer, 3 ml. of 0.87% Na_2CO_3 added and the solution shaken with air for 5–10 min. A 0.2 ml. sample of this solution was added to 2.8 ml. of 0.1*M*-phosphate buffer, pH 7.3, and the optical density at 340 $m\mu$. determined in a 1 cm. cell in a Beckman spectrophotometer, using phosphate buffer in the blank cell. Call this reading D_1 . Lactic dehydrogenase preparation (0.1 ml.) and 0.01*M*-pyruvate (0.1 ml.) were then added to both cells and the optical density again measured. The reading rapidly declines (within 1–2 min.) to a reading which may either remain stationary or decline slowly. In the latter case, readings are taken against time and extrapolated to the time of adding the dehydrogenase and pyruvate. Call this reading D_2 . Then percentage purity is 80 ($D_1 - D_2$). The factor 80 was calculated from the weight of cozymase taken, the molecular weight of cozymase and the molecular extinction coefficient found by Horecker & Kornberg (1948). D_2 , which was usually about 20% of D_1 , was much greater than the optical density of the preparation at 340 $m\mu$. before reduction. Thus, reduction with $Na_2S_2O_4$ produces substances other than Co I H_2 which absorb at 340 $m\mu$; these are possibly the reduced forms of other *N*-substituted nicotinamides present in the cozymase preparation. The presence of such substances means that methods of determining purity depending upon the increase of the optical density at 340 $m\mu$. after reduction (or upon the CO_2 produced from the $NaHCO_3$ buffer by the H^+ liberated by the reduction) will tend to give high values.

Preparation of Co I H_2 . Two methods of reduction of the cozymase were used.

(a) Sodium dithionite $Na_2S_2O_4$. The cozymase was reduced as described in the above paragraph and the solution obtained after aeration of the $NaHCO_3$ – Na_2CO_3 solution used. LePage's (1947) much simpler method was found unsuitable for this investigation, since the Co I H_2 in such solutions was found to be appreciably autooxidizable (cf. Adler, Hellström & Euler, 1936).

(b) *Enzymic.* Cozymase preparation (40 mg.), Na L-glutamate (200 mg.) and 5% nicotinamide (1 ml.) were added to 0.05M-Na₂HPO₄ (8.6 ml.) and the mixture adjusted to pH 8.3 with a few drops N-NaOH. Liver extract (0.4 ml.) was then added and the solution allowed to stand at room temperature until the reduction of cozymase, determined by adding 0.1 ml. samples to 2.9 ml. of 0.1M-phosphate buffer and measuring the optical density at 340 m μ , was a maximum (2-3 hr.). The mixture was then heated to 80° for 5 min. and the precipitate removed by centrifugation. Usually, a control solution was prepared in exactly the same way as above, omitting the cozymase. The yield of Co I H₂ was only 60-75% that of Co I used. The remainder was either unreduced or was possibly destroyed by a pyrophosphatase of the type described by Kornberg & Lindberg (1948). Nicotinamide does not inhibit this enzyme.

Co I H₂ solutions prepared by both methods suffered only slight oxidation while being kept at 4° for 4 days.

Measurement of enzyme activities

Co I H₂ oxidase system. Co I H₂ solution (sufficient to give an optical density at 340 m μ . of about 0.5-0.6 when diluted to 3.0 ml.) was added to phosphate buffer, pH 7.3, in a Beckman 1 cm. cell, the total liquid volume being 2.8 ml. and the phosphate concentration, 0.125M, except where otherwise stated. The 'blank' Beckman cell contained 2.8 ml. of the phosphate buffer. At zero time, 0.2 ml. of the enzyme preparation suitably diluted (usually 50-fold in the case of the heart-muscle preparation) in 0.125M-phosphate buffer, pH 7.3, was added to each cell and the optical density at 340 m μ . measured at approximately 20 sec. intervals for about 4 min. The readings were plotted as shown in Fig. 1, and the slope of the straight line ($=v_{\text{uncorr.}}$) measured. The phosphate concentration had an important effect on the rate of oxidation, which was optimal at 0.125M-phosphate in the case of heart-muscle preparation.

Fig. 1. Oxidation of Co I H₂ (initial concentration, 6.2×10^{-5} M) by heart-muscle preparation (0.1 mg./ml.), added at zero time. Co I H₂ prepared by enzymic reduction. Temp. 22.8-23.2°. No correction for temperature.

The reaction is very sensitive to temperature, and the accuracy of this measurement is determined by the accuracy of the temperature correction. The temperature of the solution in the cell was measured immediately before adding the heart-muscle preparation and immediately at the end of the reaction. The rise of temperature was kept small

by having the solution in the closed cell compartment of the Beckman for some time before commencing the measurement. The observed $v_{\text{uncorr.}}$ was converted to $v_{\text{corr.}}$ by the expression $\log_{10} (v_{\text{corr.}}/v_{\text{uncorr.}}) = 19,000 (T_s - T)/2.303 RT_s T$, where T is the mean observed absolute temperature, T_s is the absolute temperature chosen as the standard of the comparison (usually 293), R is the gas constant and 19,000 is the energy of activation of the reaction determined by measuring the rate of oxidation of the same preparation between 17.1 and 23.2°.

It was found that the same temperature coefficient governed the diaphorase determination (at least at low methylene blue concentrations) and it was assumed for the purposes of calculation, in the absence of any information, that it governed all the other oxidations of Co I H₂ considered in this paper. This assumption is certainly invalid in some cases, but any error caused by making it was minimized by keeping the temperature difference between different measurements in any one experiment as small as possible.

There was no need to aerate the solution during these measurements, since the amount of O₂ dissolved in water is about five times that necessary for complete oxidation of the Co I H₂, in the concentrations employed.

The heart-muscle preparation was diluted immediately before use, since its Co I H₂ oxidase was somewhat unstable in diluted solutions.

In the Figures which follow, all optical densities have been corrected to a fluid volume of 3.0 ml.

Succinic oxidase. This was determined as previously described (Slater, 1949c) except that no cytochrome *c* was added, the temperature was 25° and the diluted heart-muscle preparation was in the dangling tube, while the succinate was in the main solution. The temperature coefficient of this reaction was determined by measurements with the same heart-muscle preparation at 26.1 and 39.3°. The energy of activation, calculated from the rates at these two temperatures, was much less than that of the Co I H₂ oxidase system, viz. 7520 cal. This value, which was not affected by the addition of cytochrome *c*, is considerably different from those previously reported, viz. 11,200 by Hadidian & Hoagland (1939) and 11,500 by Thorn (1949). The value obtained in the present investigation was used only for comparing rates measured at room temperature with those at 38° and, until further measurements are made, too much significance should not be given to the actual value.

EXPERIMENTS

Components of the cytochrome system involved in the oxidation of dihydrocozymase-spectroscopic evidence

A fresh heart-muscle preparation was placed in the main compartment of a Thunberg tube and a solution of Co I H₂ (sufficient to give a final concentration of 3.3×10^{-4} M when mixed with the heart-muscle preparation) in the hollow stopper. Oxygen was removed by evacuation, refilling with oxygen-free nitrogen, repeating the evacuation and refilling several times and finally evacuating. The Co I H₂ in the hollow stopper was then mixed with the heart-muscle preparation and the mixture immediately examined under a low-dispersion visual spectro-

scope. Strong *a* and *c* bands appeared immediately; a faint *b* band appeared on further standing. All bands disappeared immediately when the tube was opened to air and shaken. Essentially the same results were obtained whether the Co I H_2 was prepared by reduction with sodium dithionite or enzymically. No bands appeared when Co I replaced the Co I H_2 .

When the same concentration of succinate was used instead of Co I H_2 , there was the immediate appearance of a definite *b* band, corresponding to about half-complete reduction, in addition to strong *a* and *c* bands. This degree of reduction of cytochrome *b* by succinate is approximately that expected from the reported oxidation-reduction potentials (Ball, 1938) and the concentration of the cytochromes in heart-muscle preparation (Slater, 1949*c*). Since the oxidation-reduction potential of cozymase (-0.29 V. at pH 7.3, calculated from Borsook, 1940) is much less than that of the cytochromes, it would be expected, purely on thermodynamic grounds, that cytochrome *b* would be completely reduced by the concentration of Co I H_2 used. The finding that cytochrome *b* is only partially (and slowly) reduced by Co I H_2 under the conditions of this experiment might be because (1) cytochrome *b* is not on the main pathway for the oxidation of Co I H_2 by heart-muscle preparation, but is concerned in a side reaction; or (2) the oxidation of Co I H_2 by heart-muscle preparation is very slow compared with that of succinate. The second possibility can be definitely excluded since, as is shown below, the Co I H_2 oxidase activity of heart-muscle preparation is of the same order as its succinic oxidase activity, when both are measured in the presence of optimal substrate. With the concentrations used in this experiment, the Co I H_2 oxidase activity must have been considerably greater than the succinic oxidase.

When the above experiment with Co I H_2 was repeated with cyanide also present in the reaction mixture, the faint *b* band slowly increased in intensity, until it eventually corresponded to complete reduction.

When succinate (final concentration, $3.3 \times 10^{-4} \text{ M}$) was added to heart-muscle preparation treated with BAL (under such conditions that the succinic oxidase system was completely inactivated) and the mixture shaken with air, a strong *b* band, but no *c* or *a* bands, was visible (cf. Slater, 1949*a*). If the same concentration of Co I H_2 was used, no bands were visible. It is shown below that the treatment with BAL inactivates the Co I H_2 oxidase system as well as the succinic oxidase system. In another experiment, heart-muscle preparation was shaken in air with BAL until all the BAL was oxidized. When this was freed from air no bands were visible (residual BAL will, under anaerobic conditions, reduce cytochromes *a*, *c* and *b*, the latter very slowly). On adding Co I H_2 , the bands of *c* and *a* appeared very

much more slowly than with untreated heart-muscle preparation.

Strong *a* and *c* bands, accompanied by a barely visible *b* band, were observed when lactate, lactic dehydrogenase, cozymase and cyanide were added to heart-muscle preparation, in the presence of air. If the lactic dehydrogenase was omitted, the bands still appeared, but much more slowly, probably due to the presence of a small amount of lactic dehydrogenase in the heart-muscle preparation (Keilin & Hartree, 1940). No bands appeared if the lactate, cozymase or cyanide were omitted. The cyanide serves a double purpose of enabling the reduction of the cozymase by the lactate-lactic dehydrogenase by removing the pyruvate formed (Green & Brosteaux, 1936), and of preventing the oxidation of the Co I H_2 through the cytochrome oxidase, which would rapidly exhaust the substrate. The *b* band was not reinforced by adding urethane, a procedure which strongly intensifies the *b* band when its reduction is, in the absence of urethane, slow compared with its oxidation.

It was found by the following experiments that the *a* band, formed by adding Co I H_2 to heart-muscle preparation, was due to both cytochromes *a* and a_3 , as is the case when succinate is the reducing agent (Keilin & Hartree, 1939).

(1) When Co I H_2 was added to heart-muscle preparation under anaerobic conditions and the tube then filled with CO, the *a* band was broadened in the direction of shorter wavelength, due to the formation of cytochrome a_3^+ -CO which absorbs at $589 \text{ m}\mu$.

(2) Heart-muscle preparation was diluted tenfold with strong sucrose syrup. After removal of air, Co I H_2 was added and the solution examined with the visual spectroscope, using blue-violet light. The γ -band at $448 \text{ m}\mu$, which Keilin & Hartree (1939) have shown to be due mainly to cytochrome a_3 , was equally as strong as when succinate was the reducing agent. When CO was admitted, this band became very faint, while there was an intensification of the previously faint band at about $430 \text{ m}\mu$, due to cytochrome *b*. This change of spectrum is due to the formation of a_3^+ -CO which absorbs at $432 \text{ m}\mu$, leaving the faint γ -band of cytochrome *a* at $452 \text{ m}\mu$ unaffected (Keilin & Hartree, 1939).

(3) A very distinct band of a_3^+ -CN at $590 \text{ m}\mu$ was visible after standing when Co I H_2 was added to heart-muscle preparation in the presence of potassium cyanide, under anaerobic conditions. This band disappeared immediately on admitting air, while the *a* and *c* bands were unaffected.

The aerobic oxidation of Co I H₂ by heart-muscle and kidney preparations

The activity of the Co I H_2 oxidase system in heart-muscle preparation may be very conveniently studied by measuring the rate of decrease of the

optical density at 340 $m\mu$., in a Beckman spectrophotometer. The actual details of the measurement are given in the Method section. The dilution of the heart-muscle preparation in this measurement was usually about 750-fold. At this dilution, the scattering of light by the insoluble heart-muscle preparation is not great and is eliminated from the measurements by adding the preparation to both the 'blank' and the 'test' cuvettes. An actual measurement is given in Fig. 1. It can be seen that a uniform rate of oxidation of Co I H_2 was maintained until nearly all the Co I H_2 was oxidized, indicating that the enzyme system must have a very high affinity for the Co I H_2 , or that the first step of the reaction series does not limit the overall reaction rate, until nearly all the Co I H_2 is oxidized.

Curve 1, Fig. 2, shows that, under the conditions of the test, Co I H_2 was oxidized very slowly until the heart-muscle preparation was added (at the first

Fig. 2. Oxidation of Co I H_2 (prepared by enzymic reduction) by heart-muscle preparation (H.M.P.) and by lactic dehydrogenase and pyruvate. Final concentrations: Co I H_2 , $8.2 \times 10^{-5}M$ (at commencement of reaction); H.M.P., 0.074 mg./ml.; pyruvate, $3.3 \times 10^{-4}M$; lactic dehydrogenase (L.D.), one-thirtieth of concentrated preparation; methylene blue (M.B.), $6.25 \times 10^{-5}M$; KCN, 0.01M; phosphate buffer, 0.125M. The 'blank' cuvette contained the appropriate amount of a solution prepared in the same way as the Co I H_2 , without the addition of the cozymase. Temp. 27.7–31.0°. No correction for temperature.

Curve	Added at			
	Zero time	1st arrow	2nd arrow	3rd arrow
1	Co I H_2	H.M.P.	—	—
2	Co I H_2 , L.D., pyruvate	M.B.	—	—
3	Co I H_2	H.M.P.	KCN	M.B.
4	Co I H_2 , KCN	H.M.P.	M.B.	—

arrow), when a rapid oxidation occurred. The optical density at 340 $m\mu$. was not brought to zero by the heart-muscle preparation (see also Fig. 1). This is due to the presence of substances in the Co I H_2 prepara-

tion other than Co I H_2 which absorb at 340 $m\mu$. The same final reading was obtained when the Co I H_2 was oxidized by lactic dehydrogenase and pyruvate ($3.3 \times 10^{-4}M$) (curve 2, Fig. 2). Since the equilibrium constant of the reaction

is very high (Euler, Adler, Gunther & Hellström, 1937), the forward reaction proceeds virtually to completion under the conditions of this experiment.

Since there is evidence (Adler *et al.* 1936; Hellström, 1937; Karrer & Benz, 1936) that the reduction of Co I by $Na_2S_2O_4$ (dithionite) to Co I H_2 proceeds through the semiquinone or half-reduced compound Co I H., it was necessary to consider the possibility that Co I H_2 is oxidized in two distinct steps:

- (1) $\text{Co I } H_2 + \frac{1}{2}O_2 \rightarrow \text{Co I H.} + \frac{1}{2}H_2O$
- (2) $\text{Co I H.} + \frac{1}{2}O_2 \rightarrow \text{Co I} + \frac{1}{2}H_2O$

Overall reaction:

Adler *et al.* (1936) and Hellström (1937) have studied in detail the properties of a yellow substance which appears transiently when Co I is reduced by $Na_2S_2O_4$ in neutral solution, and is stabilized in alkaline solution. This yellow substance, which is believed by these authors to be Co I H., has an absorption maximum at 360 $m\mu$. and shows a considerable absorption at 340 $m\mu$. (amounting to at least 39 % that of Co I H_2). Thus, if any Co I H. accumulated during the oxidation of Co I H_2 , the rate of decrease of the optical density might not be an accurate index of the rate of the overall reaction (3). The accumulation of Co I H. was tested experimentally by measuring the rate of the decline of the optical density at 340 and 380 $m\mu$. with the same heart-muscle preparation. The ratio of the two rates (0.204) was practically the same as the relative optical densities of Co I H_2 at these two wavelengths (0.212). This shows that there was no accumulation of Co I H., since this substance absorbs more than Co I H_2 at 380 $m\mu$. This was confirmed by following the optical density at 400 $m\mu$. during the oxidation of Co I H_2 . The latter absorbs very slightly at this wavelength, whereas the yellow substance absorbs strongly. Thus any accumulation of Co I H. would be revealed by an increase of the optical density at 400 $m\mu$., but no such increase was found. It is concluded that the rate of decrease of the optical density at 340 $m\mu$. is a reliable measure of the reaction (3) and enables the calculation of the Q_{O_2} (Co I H_2) of the enzyme preparations.

The finding that Co I H. does not accumulate during the course of the aerobic oxidation of Co I H_2 does not dismiss the possibility that the reaction proceeds in two single electron steps, since reaction (2) may be very much more rapid than reaction (1).

There are, in fact, good reasons for believing that the oxidation proceeds in single electron steps (Michaelis & Schubert, 1938; Luvall & Goddard, 1948).

Effect of inhibitors of cytochrome oxidase. In curve 3, Fig. 2, cyanide (0.01M finally) was added at the second arrow after about two-thirds of the Co I H₂ had been oxidized. This stopped the oxidation, which was restored by the addition of methylene blue at the third arrow. After the addition of cyanide, the optical density actually increased slightly. This is undoubtedly due to combination of oxidized cozymase (or other *N*-substituted nicotinamide compounds), either originally present in the Co I H₂ preparation or formed by the oxidation of the Co I H₂ by the heart-muscle preparation, with cyanide to give a compound which absorbs at 340 mμ. (Meyerhof, Ohlmeyer & Möhle, 1938). Co I H₂ does not react with cyanide.

In order to measure more accurately the degree of inhibition caused by the cyanide, the Co I H₂ and potassium cyanide were kept in contact for 30 min., until a stable reading was obtained (curve 4, Fig. 2). The heart-muscle preparation was then added at the first arrow. It can be seen that, in the presence of 0.01M-cyanide, the reaction is brought almost completely to a standstill. More accurate measurements with more concentrated heart-muscle preparations showed that the inhibition by 0.01M-cyanide was 99.6%. Inhibitions by lower concentrations of cyanide are shown in Table 1.

The rate of oxidation of Co I H₂ by heart-muscle preparation in the presence of cyanide and methylene blue is a measure of the diaphorase activity of the heart-muscle preparation (p. 494).

Co I H₂, prepared as described in the Methods section by reduction with Na₂S₂O₄, cannot be used for these experiments since, in the absence of any enzyme, the addition of potassium cyanide to such Co I H₂ preparations caused a rapid decrease of the optical density at 340 mμ. The reason for this is not known.

That the inhibition by cyanide is easily reversible was shown by the following experiment. Undiluted heart-muscle preparation was kept in contact with 10⁻³M-cyanide for 30 min. at room temperature (*A*). It was then diluted 1500 times for measurement of Co I H₂ oxidase activity. A control (*B*) was treated in the same way as *A* without the addition of cyanide. Additional measurements were made in which cyanide was added to the diluted heart-muscle preparation *B* to final concentrations of 10⁻³M and 6.67 × 10⁻⁷M-potassium cyanide respectively, the latter being the concentration of cyanide after dilution of *A*. The results (Table 2) clearly show that the degree of inhibition is governed only by the concentration of cyanide during the measurement of the enzyme activity and not by the concentration during standing before dilution. It was found in another experiment that the degree of inhibition by 10⁻³M-potassium cyanide was independent of the concentration of the heart-muscle preparation (over a 20-fold

Table 1. *Effect of inhibitors of cytochrome oxidase on coenzyme oxidase activity of heart-muscle preparation*

Inhibitor	Concentration (M)	Inhibition (%)		
Cyanide	6.67×10^{-7}	11		
	3.33×10^{-4}	93.9		
	5×10^{-4}	96.9		
	1×10^{-3}	98.3		
	3.33×10^{-3}	98.9		
	1×10^{-2}	99.6		
Azide	1×10^{-3}	72.2		
	5×10^{-3}	88.7		
	1×10^{-2}	91.7		
	2×10^{-2}	93.6		
	5×10^{-2}	96.0		
Hydroxylamine	3.33×10^{-3}	30		
	1×10^{-2}	63.5		
	3.33×10^{-1}	92		
Sulphide	1×10^{-4}	96.3		
	1×10^{-2}	99		
Fluoride	1×10^{-2}	7		
	3×10^{-2}	14		
	CO (%)	O ₂ (%)		
Carbon monoxide:	H.M.P.* no. 1	80	20	37
	H.M.P. no. 2†	79	21	11.6
	H.M.P. no. 2‡	95.1	4.9‡	52.4

* H.M.P. = heart-muscle preparation.

† These experiments were carried out in 0.256M-phosphate, compared with 0.125M-phosphate in all other experiments.

‡ The Co I H₂ oxidase activity in 95% N₂, 5% O₂ was the same as in air.

Table 2. *Reversibility of inhibition by cyanide of Co I H₂ oxidase*

(See text for details.)

Heart-muscle preparation	Concentration of KCN (M)		Co I H ₂ oxidase ($v_{\text{corr.}}$)	Inhibition (%)
	During standing	During measurement of Co I H ₂ activity		
B	0	0	0.0350	—
B	0	6.67×10^{-7}	0.0310	11
B	0	10^{-3}	0.00052	98.5
A	10^{-3}	6.67×10^{-7}	0.0311	11

range of concentrations). It follows from these two experiments that the inhibition by cyanide is completely reversible by dilution.

Table 1 shows that other inhibitors of cytochrome oxidase, viz. azide, hydroxylamine, sulphide and CO also inhibit the oxidation of Co I H₂. Fluoride, which has only a very slight effect on cytochrome oxidase (Borei, 1945), also had little effect on the Co I H₂ oxidation.

The effect of CO on the Co I H₂ oxidase systems of two heart-muscle preparations, which differed considerably in Co I H₂ oxidase activity, is shown in Table 1. The more active preparation (no. 1) was considerably more susceptible to inhibition by CO. The degree of inhibition by CO is usually expressed by the value of K , which equals $\frac{n}{1-n} \frac{[\text{CO}]}{[\text{O}_2]}$, where n = activity of system in presence of CO compared with that of control, and $[\text{CO}]$ and $[\text{O}_2]$ are the partial pressures of CO and O₂ in the gas mixture. The K of heart-muscle preparation no. 1 was 6.8 and that of no. 2 was 17.7.

The effect of bright light on the inhibition by CO is shown in Fig. 3. In the experiment shown in curve 3, the solution was alternately kept in the dark and placed in the bright light of a mercury vapour lamp. Whenever the solution was in the dark, the oxidation of the Co I H₂ followed the same course as the inhibited reaction kept in the dark throughout (curve 2), while whenever it was placed in the light, the curve is parallel to the uninhibited reaction (curve 1). Thus, bright light completely reverses the CO inhibition. Four successive reversals by light of the inhibition are shown. It is apparent that both the inactivation by CO and the reversal by light are very rapid processes. Curve 1, Fig. 3, shows that the light from the mercury vapour lamp had no effect on the rate of oxidation in air.

The easily reversible inhibition by low concentrations of cyanide, the light sensitivity of the CO inhibition and the fact that the relative susceptibility of the Co I H₂ oxidation to the inhibitors in Table 1 is the same as that of cytochrome oxidase leaves little doubt that cytochrome oxidase is concerned in the oxidation of Co I H₂. The Co I H₂ oxidase system in heart-muscle preparation would be convenient for the determination of the photochemical absorption spectrum of the CO-cytochrome oxidase compound.

Effect of BAL. It has been shown previously (Slater, 1948, 1949a) that treatment of heart-muscle preparation with 2:3-dimercaptopropanol (BAL) in

Fig. 3. Inhibition of Co I H₂ oxidase by CO and reversal by light. Co I H₂ prepared by enzymic reduction. Final concentrations, H.M.P., 0.029 mg./ml.; Co I H₂, 4.8×10^{-5} M (at commencement of reaction); phosphate buffer, 0.256 M. The reactions were carried out in 2 cm. cells with a narrow neck and close-fitting stopper. When filled (total vol. = 5.0 ml.), there was only a small gas space. The gas mixture was bubbled through the solution of Co I H₂ in phosphate buffer in the cell for some time. At zero time, the H.M.P. was added, the cell transferred to the Beckman compartment and readings taken with time. The effect of light was studied by removing from the Beckman and placing in the light from a mercury vapour lamp focused by two lenses for 1 min. periods. Periods when the cells were in the dark are shown by full lines on the graph, those in the light by dotted lines. Gas phases: curve 1, air; curves 2 and 3, 95.1% CO, 4.9% O₂. The measurements which were made between 22.2 and 22.9° have not been corrected for temperature.

air causes the destruction of a factor essential for the succinic oxidase system, acting between cytochromes b and c. Fig. 4 shows that treatment of heart-muscle

preparation with 0.02M-BAL for 15 min. at 39° inactivates the Co I H₂ oxidase system by 99% (cf. curves 2 and 3), without affecting the diaphorase activity, measured by adding cyanide and methylene blue (curves 3, 4 and 5).

A comparison of curve 1, obtained with untreated heart-muscle preparation and curve 2, which serves as a control for the BAL treatment, shows that treatment of the heart muscle for 15 min. at 39° in the absence of BAL has some effect on the Co I H₂ oxidase activity. After such treatment, there is some lag in reaching the maximal rate of oxidation after

Fig. 4. Effect of treatment with BAL on Co I H₂ oxidase and diaphorase activity of heart-muscle preparation (H.M.P.). Co I H₂ prepared by enzymic reduction. H.M.P. 1, untreated. H.M.P. 2, shaken in air at 39° for 15 min., then diluted. H.M.P. 3, shaken with 0.02M-BAL in air at 39° for 15 min., then diluted ('General procedure' of Slater, 1949a). Final concentrations: H.M.P., 0.037 mg./ml.; methylene blue (M.B.), 6.25×10^{-5} M; KCN, 0.01M; phosphate buffer, 0.125M. The 'blank' cuvette contained the appropriate amount of a solution prepared in the same way as the Co I H₂, without the addition of the cozymase. Measurements were carried out at temperatures between 25.5 and 30.2° and the curves corrected to 20° by appropriate adjustment of the time scale.

Curve	Added at		
	Zero time	1st arrow	2nd arrow
1	Co I H ₂ , H.M.P. 1	—	—
2	Co I H ₂ , H.M.P. 2	—	—
3	Co I H ₂ , H.M.P. 3	M.B.	—
4	Co I H ₂ , KCN	H.M.P. 3	M.B.
5	Co I H ₂ , KCN	H.M.P. 2	M.B.

adding the heart-muscle preparation to the Co I H₂. The maximal rate is, however, usually only about 10% less than that given by an untreated heart-muscle preparation (in Fig. 4, it has been affected to a somewhat greater degree). The lag was much more pronounced if the warming to 39° was carried out at low phosphate concentrations. Shaking the heart-muscle preparation in air at 19° for 1 hr. or at 25° for 45 min. had no effect on the Co I H₂ oxidase activity. Treatment with BAL under these conditions had the

same effect on the Co I H₂ oxidase system as for 15 min. at 39°.

For these experiments, Co I H₂, prepared by Na₂S₂O₄ reduction, was not as satisfactory as that prepared by enzymic reduction, since BAL treatment of heart-muscle preparation did not completely stop the oxidation measured by the decline of the optical density at 340 mμ. The residual oxidation amounted to 5–20% of the initial rate. This is very likely due to bisulphite derived from the Na₂S₂O₄ (Adler *et al.* 1936; Hellström, 1937) in these Co I H₂ preparations, since Co I H₂ prepared enzymically behaved in the same way when sodium bisulphite (6.4×10^{-5} M) was added.

Fig. 5. Comparison of the inactivation of the succinic oxidase system and of the Co I H₂ oxidase system in heart-muscle preparation by treatment with different concentrations of BAL. 1 ml. of heart-muscle preparation (40 mg./ml.) treated with various concentrations of BAL in a total volume of 2 ml. for 45 min. at 25.7° with shaking in air. Concentration of phosphate buffer, 0.1 M. Heart-muscle preparation diluted to 0.48 mg./ml. for measurement of succinic oxidase activity and to 0.053 mg./ml. for measurement of Co I H₂ oxidase activity. Succinic oxidase activity measured at 25.9°. Co I H₂ oxidase activity measured at temperatures between 24.2 and 27.4° and corrected to same temperature for calculation of percentage inhibition. Q_{O_2} (μl. of O₂/mg. of fat-free dry wt./hr.) of controls at 25.9°: succinic oxidase, 291; Co I H₂ oxidase, 305; ●—●—, Co I H₂; ○—○—, succinic oxidase.

In Fig. 5, the effect of different concentrations of BAL on the Co I H₂ and succinic oxidase systems in heart muscle are compared. The two systems require approximately the same concentration of BAL for complete or nearly complete inactivation. The Co I H₂ oxidase system is appreciably more sensitive than the succinic oxidase to low concentrations of BAL.

Two possible explanations of the effect of BAL on the Co I H₂ oxidase system were considered: (1) the BAL-sensitive component of the succinic oxidase system is also a component of the Co I H₂ oxidase system; (2) coincident with the destruction of the BAL-sensitive component, there is an alteration of

the structure of the particles of the heart-muscle preparation which enables the diaphorase to dissociate from the particles so that much of the diaphorase is in solution and no longer readily accessible to the other components of the system. Although the fact that the Co I H_2 oxidase system was rather more susceptible than the succinic oxidase system to BAL made it unlikely, it was necessary to consider this second possibility, since it is known that diaphorase is more readily dissociated from the insoluble particles than are other components. If this second explanation is correct, it should be possible to re-activate the system after treatment with BAL by adding a large excess of purified diaphorase. Experiments along these lines are summarized in Table 3.

Table 3. *Effect of diaphorase and cytochrome c on the inactivation of Co I H_2 oxidase by BAL*

(Exp. 1. BAL treatment consisted of shaking concentrated heart-muscle preparation with 0.029 M-BAL for 80 min. at 15°. Co I H_2 oxidase activity of 1500-fold diluted heart-muscle preparation (0.023 mg./ml.) measured in 0.13 M-phosphate. Diaphorase added equalled 13.5 times that present in the heart-muscle preparation. Activity after addition of diaphorase corrected for rate of oxidation with diaphorase alone (viz. 0.0127). Co I H_2 prepared by $Na_2S_2O_4$ reduction.

(Exp. 2. BAL treatment consisted of shaking concentrated heart-muscle preparation with 0.0025 M-BAL for 45 min. at 25°. Co I H_2 oxidase activity of 375-fold diluted heart-muscle preparation (0.093 mg./ml.) measured in 0.022 M-phosphate. Diaphorase added equalled eight times that present in the heart-muscle preparation. Added cytochrome c, 3.8×10^{-5} M. Activity after addition of diaphorase corrected for rate of oxidation with diaphorase alone (0.0033). Co I H_2 prepared by enzymic reduction.)

	Co I H_2 oxidase activity ($v_{corr.}$)		Inactivation (%)
	Control	BAL treated	
Exp. 1			
No addition	0.0208	0.0046	78
+ diaphorase	0.0188	0.0049	73
Exp. 2			
No addition	0.0298	0.0163	45.1
+ diaphorase	0.0532	0.0288	45.9
+ cytochrome c	0.0340	0.0186	45.3
+ diaphorase	0.0557	0.0308	44.7
+ cytochrome c			

It was necessary to limit the amount of diaphorase added, since isolated diaphorase is appreciably autooxidizable, and oxidizes Co I H_2 without any other addition. Measurements were corrected for this 'blank' oxidation. In Exp. 1, which was carried out in 0.13 M-phosphate buffer, the diaphorase activity of the added diaphorase solution was 13.5 times that of the heart-muscle preparation. In this experiment, added diaphorase had little effect on either the control or BAL treated heart-muscle preparation.

The Co I H_2 oxidase system is much less active at low phosphate concentrations than in 0.13 M-phosphate. Exp. 2 shows the effect of diaphorase on the inactivation caused by BAL at 0.022 M-phosphate. In this experiment, cytochrome c (3.8×10^{-5} M) was also added since it was possible that, at this low phosphate concentration, the accessibility of the cytochrome c and not of the diaphorase to the rest of the system was limiting the activity of the Co I H_2 oxidase (cf. the effect of cytochrome c on the activity of the succinic oxidase system at low phosphate concentrations, Slater, 1949*d*). In this case, eight times as much diaphorase as present in the heart-muscle preparation was added (the actual amount added was greater than in Exp. 1, but more heart-muscle preparation was used). It is apparent that, although diaphorase caused a considerable increase in activity of the control at the low phosphate concentration used in this experiment, it did not have any effect on the inactivation caused by the BAL. Similarly, cytochrome c had little effect on the inactivation; it also has no effect on the inactivation caused by BAL of the succinic oxidase system (Slater, 1949*a*).

The experiments summarized in Table 3 make it very unlikely that BAL acts on the Co I H_2 system by causing the dissociation of diaphorase from the particles of the heart-muscle preparation.

Fig. 6. Effect of added cytochrome c on Co I H_2 oxidase activity of kidney preparation. Final concentrations, kidney preparation, 0.165 mg./ml.; phosphate buffer, 0.045 M. Reactions carried out between 19.6 and 22.8°; rates corrected to 20°.

The Co I H_2 oxidase system behaved in the same way as the succinic oxidase system in the following respects: (1) treatment of the heart-muscle preparation with BAL in the absence of air had a negligible effect on the enzyme activities; (2) the effect of the BAL was not due just to the hydrogen peroxide produced during the oxidation of BAL, since hydrogen peroxide formed by notatin-glucose had a much smaller effect on the system.

Effect of added cytochrome c. Added cytochrome *c* did not have a very marked effect on the Co I H_2 oxidase activity of heart-muscle preparation, even at low phosphate concentrations, when added cytochrome *c* has its maximal effect on the succinic oxidase system (Slater, 1949*d*). A very marked effect of added cytochrome *c* was, however, obtained with kidney preparation (Fig. 6), 3.8×10^{-5} M-cytochrome *c* being necessary for the maximum effect. The succinic oxidase system in kidney is likewise strongly stimulated by the addition of cytochrome *c* (Keilin & Hartree, 1940, 1945, 1949*a*; Slater, 1949*c*).

*The anaerobic oxidation of Co I H_2
by fumarate*

Neither fumarate (0.027 M) nor malonate (0.0133 M) had any effect on the aerobic oxidation of Co I H_2 by heart-muscle preparation.

However, the finding that the BAL-sensitive factor was common to both the succinic oxidase and Co I H_2 oxidase systems and that, although cytochrome *b* was not in the main pathway for the oxidation of Co I H_2 , a faint *b* band appeared when Co I H_2 was added to heart-muscle preparation suggested some link between the two oxidase systems. If there is such a link between the two systems, one would expect that the more positive fumarate would be able to oxidize the very negative Co I H_2 . Indeed, that such a reaction does occur was suggested by Dewan & Green (1937*a*) who found that, under anaerobic conditions, fumarate brings about the oxidation of β -hydroxybutyrate and that cozymase is required for this reaction. A direct observation of the oxidation of Co I H_2 by fumarate has not, however, hitherto been reported in the literature. Accordingly, the effect of fumarate on Co I H_2 and heart-muscle preparation under anaerobic conditions was investigated.

Fig. 7, curve 1, shows that when Co I H_2 was added to heart-muscle preparation in the absence of air, no oxidation of the Co I H_2 occurred. As soon as fumarate was added, however, the Co I H_2 was oxidized.

This oxidation of Co I H_2 by fumarate could also be studied in the presence of air by adding cyanide to inhibit almost completely the aerobic oxidation of the Co I H_2 and then adding fumarate (Fig. 8). The slow oxidation in the presence of cyanide alone was subtracted from the rate obtained after adding fumarate in order to obtain the rate due to fumarate alone.

Reduction of cozymase by malic dehydrogenase and malate formed from the fumarate by the action of fumarase was insignificant. The thoroughly washed heart-muscle preparation probably contains very little malic dehydrogenase.

The following conclusions may be drawn from the experiments summarized in Figs. 7 and 8 and from other experiments:

(1) When the aerobic oxidation of Co I H_2 is prevented either by removing oxygen or by adding cyanide, the Co I H_2 may be oxidized by fumarate.

Fig. 7. Anaerobic oxidation of Co I H_2 by fumarate. These experiments were carried out in a Thunberg tube with optically plane faces. Light path = 0.5 cm. There was no blank cuvette; thus the measurements of optical density included light absorbed and scattered by the Thunberg vessel, by the heart-muscle preparation and the other additions as well as by the Co I H_2 . The tubes with contents shown in second column below were evacuated and contents of hollow stopper added at arrow. Final concentrations: heart-muscle preparation, 0.46 mg./ml.; phosphate buffer, 0.13 M; Co I H_2 (prepared by $\text{Na}_2\text{S}_2\text{O}_4$ reduction), 6×10^{-5} M at commencement of reaction; fumarate, 0.027 M; malonate, 0.054 M. BAL treatment consisted of shaking concentrated heart-muscle preparation with 0.029 M-BAL for 80 min. at 15°. Measurements were carried out at temperatures between 20 and 25° and corrected to 20° by appropriate adjustment of time scale.

Curve	In main compartment	In hollow stopper
1	Co I H_2 , H.M.P.	Fumarate
2	Co I H_2 , H.M.P., fumarate	Malonate
3	Co I H_2 , BAL-treated H.M.P.	Fumarate

(2) This anaerobic oxidation of Co I H_2 by fumarate is very slow compared with the aerobic oxidation. The mean rate of oxidation by fumarate in the presence of cyanide was found to be 2% that of the oxidation of Co I H_2 or of succinate at the temperature of the experiments (23.9–27.6°).

(3) Although the reaction is slow, the anaerobic oxidation of Co I H_2 by fumarate proceeds to completion. Thus the final value in curve 3, Fig. 8, was not decreased by adding pyruvate and lactic dehydrogenase. This final value is slightly higher than that obtained by aerobic oxidation (curve 1), probably because of the reaction between cyanide and cozymase (p. 489).

(4) The anaerobic oxidation was inhibited 83 % and the oxidation in the presence of cyanide 96 % by the addition of malonate in double the concentration of fumarate. This indicates that succinic dehydrogenase is concerned in the reaction.

(5) Treatment of the heart-muscle preparation with BAL inactivates the anaerobic oxidation by 60 %. The oxidation still remaining after the treatment with BAL was also strongly inhibited by malonate.

Fig. 8. Oxidation of Co I H_2 in presence of heart-muscle preparation (H.M.P.) and cyanide by fumarate. Co I H_2 prepared by enzymic reduction. H.M.P. 1, shaken in air at 39° for 15 min., then diluted. H.M.P. 2, shaken with 0.02M -BAL in air at 39° for 15 min., then diluted. Final concentrations: H.M.P., 0.185 mg./ml. ; Co I H_2 , $5.6 \times 10^{-5}\text{M}$ at commencement of experiment; KCN, 0.01M ; fumarate, 0.01M ; malonate, 0.02M ; pyruvate, $3.3 \times 10^{-4}\text{M}$; lactic dehydrogenase (L.D.), one-thirtieth of concentrated preparation; phosphate buffer, 0.125M . The 'blank' cuvette contained the appropriate amount of a solution prepared in the same way as the Co I H_2 , without the addition of the cozymase. Measurements were carried out at temperatures between 24.4 and 28.8° and corrected to 20° by appropriate adjustment of the time scale.*

The role of cytochrome *b* in this anaerobic reaction could be seen with the visual spectroscope. The faint *b* band obtained when Co I H_2 was added to heart-muscle preparation in the absence of air disappeared immediately on the subsequent addition of fumarate.

Diaphorase activity of heart-muscle preparation

If it is assumed that diaphorase is the first member of the respiratory chain with which Co I H_2 reacts

(p. 495), the aerobic oxidation of Co I H_2 may be described by two reactions (each of which is probably the sum of a number of reactions), viz.

where *D* and DH_2 are oxidized and reduced diaphorase respectively. The rate of reaction (1) can be determined independently, since DH_2 is oxidized by methylene blue (*MB*)

Thus the oxidation of Co I H_2 by methylene blue, in the presence of cyanide to stop reaction (2), is due to reaction (1), followed by reaction (3), followed by the reoxidation of methylene blue. Under the conditions of the experiments, this latter reaction was rapid (as shown by following the reaction at $650\text{ m}\mu$., at which wavelength methylene blue absorbs strongly). If reaction (3) is made very fast compared with reaction (1), the overall reaction will be a measure of reaction (1). It would be expected that reaction (3) could be made very rapid by increasing the methylene blue concentration sufficiently. However, it was found that the overall reaction continued to increase with increasing methylene blue concentration at concentrations of the latter which strongly absorbed at $340\text{ m}\mu$. and which oxidized Co I H_2 appreciably in the absence of any enzyme. This difficulty was overcome by extrapolating rates obtained at different methylene blue concentrations (corrected for the oxidation in the absence of enzyme) to infinite methylene blue concentration by the method of Lineweaver & Burk (1934) (cf. Slater, 1949*e*), since it was found that the relation activity: methylene blue concentration was a rectangular hyperbola. This extrapolated velocity must represent the rate of reaction (1). In the case of heart-muscle preparation this was found to be 3–4 times the Co I H_2 oxidase activity, measured in the absence of added cytochrome *c*. With the kidney preparation, the rate of reaction (1) was 2.2 times that of the aerobic oxidation of Co I H_2 , in the presence of excess cytochrome *c*.

DISCUSSION

Components of the dihydrocozymase oxidase system

On the basis of the above experiments, it is suggested that the Co I H_2 oxidase system consists of the

*

Curve	Added at			
	(Zero–30) (min.)	Zero time	1st arrow	2nd arrow
1	—	Co I H_2 , H.M.P. 1	—	—
2	Co I H_2 , KCN	H.M.P. 1	—	—
3	Co I H_2 , KCN	H.M.P. 1	Fumarate	L.D., pyruvate
4	Co I H_2 , KCN	H.M.P. 1, fumarate	Malonate	—
5	Co I H_2 , KCN	H.M.P. 2, fumarate	Malonate	—
6	—	Co I H_2 , H.M.P. 2	—	—

following components, the arrows denoting the pathway taken by the hydrogen atoms or electrons on their way from substrate to oxygen.

The addition of methylene blue enables the reaction to by-pass all the components except diaphorase. The factor is the BAL-sensitive component of the system and is probably the same as the factor previously found necessary for the oxidation of succinate (Slater, 1948, 1949*a*). This proposed scheme does not, of course, exclude the possibility that further components, not yet identified, may also be required (cf. Slater, 1949*a*). In particular, the possible role of the substance responsible for the *e* band (Keilin & Hartree, 1949*b*) of cytochrome has not yet been investigated.

We shall now consider, in turn, the evidence for the participation of each of these components.

Diaphorase. The finding that there exists in tissues an enzyme which acts as a carrier between Co I H₂ and methylene blue (Dewan & Green, 1937*b*, 1938; Adler *et al.* 1938; Straub, 1939) strongly suggested, by analogy with other dehydrogenases, that diaphorase acts *in vivo* by acting as a carrier between Co I H₂ and those members of the respiratory chain with a higher potential. Lockhart & Potter (1941) considered, on the other hand, that 'the participation of diaphorase in the cytochrome reduction remains to be shown, since its mere presence in the complete system is not enough to warrant the conclusion that it is an essential link in the transport mechanism between Co I H₂ and cytochrome *c*'. However, since diaphorase mediates the oxidation of Co I H₂ by methylene blue, it is presumably reduced by Co I H₂. The objection of Lockhart & Potter (1941) implies then that reduced diaphorase might be oxidized *in vivo* by a mechanism not involving the cytochrome system. Since the aerobic oxidation of Co I H₂ is completely sensitive to cyanide or to treatment with BAL, this hypothetical mechanism cannot be involved in the aerobic oxidation, and the function of diaphorase must be in some anaerobic oxido-reduction, which should be sufficiently rapid to account for the high diaphorase activity of various tissues. Since no such reaction has been demonstrated, it seems more likely that the function of diaphorase is in the aerobic oxidation of Co I H₂. This is supported by the following findings in the present investigation: (1) The rate of reduction of diaphorase in heart-muscle and kidney preparations by Co I H₂, calculated as described above, is sufficient to account for the very rapid aerobic oxidation of Co I H₂. (2) The Co I H₂ oxidase activity of heart-muscle preparation was greatly reduced at low phosphate concentrations, probably due to an im-

paired mutual accessibility of the components of the Co I H₂ oxidase system on the particles of the enzyme preparation. At these low phosphate con-

centrations, the system was considerably reactivated by the addition of a highly purified diaphorase solution. This reactivation resembles that produced by cytochrome *c* on the succinic oxidase system at low phosphate concentrations (Slater, 1949*d*) and strongly suggests that diaphorase is a component of the Co I H₂ oxidase system. However, the possibility that the diaphorase solution is, in this experiment, acting like the indifferent proteins discussed by Keilin & Hartree (1949*a*) cannot be excluded.

Factor. The conclusion that the BAL-sensitive factor previously described is a component of the Co I H₂ oxidase system, acting between diaphorase and cytochrome *c*, is supported by the following findings. (1) The fact that isolated diaphorase does not reduce cytochrome *c* (Lockhart & Potter, 1941, confirmed in the present investigation) is suggestive, although not conclusive, evidence that an additional catalyst is required *in vivo*. (2) Treatment of a heart-muscle preparation with BAL, in the presence of air, inactivated the Co I H₂ oxidase system by 99%, without affecting the diaphorase activity, and the system was not reactivated by the addition of diaphorase or cytochrome *c*. The cytochrome *c* oxidase activity of heart-muscle preparation is not affected by this treatment (Slater, 1949*a*). (3) The rate of reduction of added cytochrome *c* by Co I H₂ in the presence of heart-muscle preparation is almost completely inhibited by the BAL treatment (Slater, 1950). Similarly, the rate of reduction of endogenous cytochrome *c* of heart-muscle preparation by Co I H₂ was greatly reduced by the BAL treatment. (4) The demonstration of the anaerobic oxidation of Co I H₂ by fumarate and the finding that this reaction is considerably inactivated by BAL indicates that the BAL-sensitive factor must act as a link between the succinic and Co I H₂ oxidase systems. This reaction is further discussed on p. 496.

Cytochrome *c*. There is a considerable body of evidence in the literature implicating cytochrome *c* in the oxidation of Co I H₂. That added cytochrome *c* is reduced by Co I H₂ in the presence of tissue preparations was shown by Adler *et al.* (1938), Hawthorne & Harrison (1939), Straub (1940*b*), Lockhart & Potter (1941). The catalytic effect of added cytochrome *c* on the oxidation of Co I H₂ or of those substrates which require cozymase for their oxidation was reported by Hawthorne & Harrison (1939), Straub (1940*b*), Lockhart & Potter (1941) and Okuniki & Yakushiji (1940).

The present work supports these conclusions and adds the further evidence that the endogenous cytochrome *c* in the heart-muscle preparation is very rapidly reduced by the Co I H_2 . It was considered important to investigate this point since some substances, e.g. ascorbic acid, rapidly reduce cytochrome *c* in solution, but not the endogenous cytochrome *c* of the heart-muscle preparation.

The finding, in the present investigation, that Co I H_2 , reduced either by sodium dithionite or by a dehydrogenase system, rapidly reduces the cytochrome *c* in the heart-muscle preparation does not agree with Dewan & Green (1938), who observed a reduction of cytochromes *b* and *a*, but not of *c* (cf. also Green, 1936). The explanation of this difference between the results of Green and the present investigation is not clear.

Dewan & Green (1938) did not obtain any stimulation of the oxidation of triosephosphate, lactate or malate by the addition of cytochrome *c*. This is understandable, since it is probable that the enzyme preparation contained sufficient cytochrome *c* not to limit the rate of oxidation. Hawthorne & Harrison (1939) found a stimulation by cytochrome *c* only at low enzyme concentrations. In the present study, cytochrome *c* had relatively little effect on the Co I H_2 oxidase activity of heart-muscle preparation even at low phosphate concentrations, where it was found to have its maximal effect in the succinic oxidase system (Slater, 1949*d*). However, it was strongly active in stimulating the Co I H_2 oxidase of kidney preparation, increasing the activity by 11-fold in one experiment.

Cytochromes a and a_3 . The observation made by Dewan & Green (1938) and in the present investigation that cytochrome *a* is rapidly reduced by Co I H_2 and rapidly oxidized by air is strong evidence that cytochrome *a* is concerned in the reaction. That it acts after cytochrome *c*, as postulated, is less certain, but this is the most likely possibility, since cytochrome *a* has an oxidation-reduction potential 0.03V. higher than cytochrome *c* (Ball, 1938) and it is closely associated with cytochrome a_3 (Keilin & Hartree, 1939). It is not possible, however, to exclude the possibility that the positions of *a* and *c* are interchanged or even that *a* and a_3 are on alternative pathways for the passage of hydrogen atoms or electrons from one of the members of the respiratory chain with lower potential to oxygen.

It was shown that the *a* band was double, being composed both of cytochrome *a* and a_3 . Keilin & Hartree (1939) have shown that cytochrome a_3 is probably identical with cytochrome oxidase. Cytochrome oxidase is certainly concerned in the oxidation of Co I H_2 (p. 490).

Cytochrome b not a component of the Co I H_2 oxidase system. Reasons have been given above (p. 487) for believing, in opposition to Dewan & Green (1938),

that cytochrome *b* is not on the main pathway for the oxidation of Co I H_2 . This is supported by the slowness of the oxidation of Co I H_2 by fumarate; if cytochrome *b* were rapidly reduced by Co I H_2 , the latter would be rapidly oxidized by fumarate, since the oxidation of reduced cytochrome *b* by fumarate is a fast reaction. Hawthorne & Harrison (1939) agreed with Dewan & Green (1938) that the oxidation of Co I H_2 by tissues normally proceeded through cytochromes *a* and *b*, without involving cytochrome *c*, since they obtained a rapid oxidation by a preparation which they consider was freed from cytochrome *c* by repeated isoelectric precipitation. It is, however, very doubtful if the cytochrome *c* was removed by this procedure. Okuniki & Yakushiji (1940) also believe that cytochrome *b* is required, but their evidence is not at all convincing. They found a slight stimulation of the oxygen uptake when Yakushiji & Mori's (1937) preparation of cytochrome *b* was added to an incomplete system, but it is not generally accepted that Yakushiji & Mori's (1937) preparation actually contains any cytochrome *b* (Keilin & Hartree, 1939). Potter & Lockhart (1939) and Lockhart & Potter (1941) found that a heart-muscle preparation obtained by precipitation at pH 4.6 did not catalyse the reduction of cytochrome *c* by Co I H_2 , although it reduced methylene blue, suggesting that some factor operated between diaphorase and cytochrome *c* and thought that this might be cytochrome *b*. However, such acid treatment affects the physical properties of heart-muscle preparation (Keilin & Hartree, 1940, 1949*a*) and this physical alteration would be quite sufficient to explain the inactivation. It is unlikely that cytochrome *b* is affected by this treatment since the rate of reduction of methylene blue by succinate is not greatly inhibited (Keilin & Hartree, 1940) and there is evidence that cytochrome *b* is concerned in this reaction (Ball, Anfinsen & Cooper, 1947; Slater, 1949*c*).

The fact that cytochrome *b* is not concerned in the oxidation of Co I H_2 draws attention to the specific association of cytochrome *b* with the oxidation of succinate and increases the evidence that succinic dehydrogenase and cytochrome *b* are identical (cf. Slater, 1949*c*). This evidence is, in the case of animal tissues, all of a negative nature, but Pappenheimer & Hendee's (1949) recent studies with diphtherial cytochrome *b* strongly suggest that the two are identical in this organism.

Anaerobic oxidation by fumarate. The Co I H_2 and succinic oxidase systems may be described in the one scheme as follows:

It is known that the reaction between succinate and cytochrome *b* is easily reversible. If the reaction

between cytochrome *b* and the factor is also reversible to a certain extent, the slow reduction of cytochrome *b* by Co I H_2 and the anaerobic oxidation of Co I H_2 by fumarate are understandable, since in the absence of oxygen and the presence of fumarate, the reaction

can take place.

The inhibition of this reaction by malonate, which affects the reaction between cytochrome *b* and fumarate, and by BAL treatment, which destroys the factor, is in agreement with this series of reactions. The fact that inactivation by BAL treatment is not complete suggests, however, that the oxidation of Co I H_2 by fumarate proceeds not only by the reaction shown, but also by a BAL-insensitive pathway. The rate of this BAL-insensitive pathway is only about 0.7% that of the aerobic oxidation of Co I H_2 . In fact, although BAL treatment practically completely inactivates the aerobic oxidation, the residual aerobic oxidation, shown in curve 6 of Fig. 8, is nevertheless somewhat more rapid than the residual anaerobic oxidation measured by the differences between curves 2 and 5. It is quite possible that many reactions of this order of velocity may occur, for example diaphorase may be able slowly to react with cytochrome *b* directly. Fumarate hydrogenase, isolated by Fischer & Eysenbach (1937) from yeast, cannot be responsible for this oxidation since the BAL-insensitive reaction, as well as the BAL-sensitive reaction, was inhibited by malonate. Malonate has no effect on fumarate hydrogenase.

Since Co I H_2 has a lower potential than cytochrome *b* it must eventually completely reduce the cytochrome *b* if the two are capable of reaction. Actually complete reduction was obtained on standing, only when both cyanide was added and as much oxygen as possible was removed. In the anaerobic experiments in the absence of cyanide, the factor would be very slightly oxidized by traces of oxygen remaining. If the factor has a potential of 0.11 V., i.e. half-way between that of cytochromes *b* (−0.04 V.) and *c* (0.26 V.) and is present in the same concentration as *b*, it can be calculated that if only 1% of the factor is in the oxidized form the maximum reduction of the cytochrome *b* will be 24%. Similarly, a very slight cyanide-stable oxidation in the presence of oxygen and cyanide would be sufficient to prevent complete reduction of the cytochrome *b* in the aerobic experiments. In addition, under these conditions, the cytochrome *b* is slightly autoxidizable (Keilin & Hartree, 1939).

It can also be calculated that the ratio of the rate of the BAL-sensitive oxidation by fumarate to the rate of aerobic oxidation of succinate (which is completely BAL sensitive) should be 1.27% if the factor

has an oxidation-reduction potential of 0.11 V. The actual ratio found was about 1.3%. In this calculation, the following assumptions were made: (1) in the anaerobic reaction, the reduction of oxidized cytochrome *b* by reduced factor will be the rate determining step; (2) in the aerobic oxidation no reaction is limiting, so that, at the steady state, half the cytochrome *b* and half the factor will be in the reduced form; (3) the thermodynamic equations developed for reactions in solution can be applied without alteration when the reactants are attached to the colloidal particles of the heart-muscle preparation. The first of these assumptions is very probable; there is no evidence for the second, but considerable departures from the conditions assumed do not affect the final answer appreciably. The existence of this anaerobic oxidation of Co I H_2 by fumarate, its rate and its sensitivity to BAL furnishes further evidence for the existence of the factor with the properties described in this and the previous papers (Slater, 1948, 1949*a*).

The physiological significance of the anaerobic reaction is somewhat doubtful. Its relative slowness indicates that, except possibly under some special circumstances, it cannot play a very important role quantitatively in the metabolism of the cell even under anaerobic conditions. Certainly, it cannot of itself, play any role aerobically because, under these conditions, the passage of hydrogen atoms (or electrons) to fumarate cannot increase the rate of oxidation, since they enter a 'blind alley' and have to come out again if they are to reach oxygen (cf. Ball's (1942) argument against the participation of the reaction $\text{oxalacetate} \rightleftharpoons \text{malate}$ in the oxidation of Co I H_2 and also Potter, 1940*a*). Thus this reaction provides no support for the Szent-Gyorgyi theory of tissue respiration. In fact, heart-muscle preparation contains no fumarate and neither added fumarate nor malonate has any effect on the aerobic oxidation. Thus it must be concluded in agreement with Straub (1940*b*), Potter (1940*b*) and Potter & Albaum (1943) that the oxidation of Co I H_2 is not brought about by the Szent-Gyorgyi mechanism.

Co I H_2 oxidase and succinic oxidase systems. While there are important differences between the Co I H_2 oxidase and succinic oxidase systems, e.g. the latter, but not the former, is inhibited by malonate and only the latter involves cytochrome *b*, the resemblances between the two systems as they exist on the particles of the heart-muscle and kidney preparations are perhaps more striking than their differences. Thus both systems are inhibited reversibly by the powerful poisons of cell respiration such as potassium cyanide, hydrogen sulphide and azide and the inhibitions by carbon monoxide are reversed by light. Similarly, the two systems are affected by BAL in the same way. The difference in behaviour of the succinic oxidase system in heart-muscle and kidney prepara-

tions to added cytochrome *c* is repeated with the Co I H₂ oxidase system. Both systems are affected in the same way by the concentration of phosphate.

Even quantitatively the two systems have the same order of activity. The mean Q_{O_2} (μ l. of oxygen/mg. of fat-free dry wt./hr.) of the Co I H₂ oxidase system, calculated from the rate of decline of the optical density at 340 m μ ., the molecular extinction coefficient of Co I H₂ and the equation

was found to be 131 at 20°. If the temperature coefficient found between 17.1 and 23.2° holds up to 38°, as is probable, this Q_{O_2} will be 870 at 38°. This may be compared with the average Q_{O_2} for the succinic oxidase system and of the cytochrome oxidase portion previously reported (Slater, 1949*c*), viz. 500 (in absence of added cytochrome *c*) and 3200 respectively at 38°. The corresponding figures for the kidney preparation are Co I H₂ oxidase (with excess cytochrome *c*), 40 at 20°, equivalent to 270 at 38°; succinic oxidase (with excess cytochrome *c*), 200 at 38°; cytochrome oxidase, 1380 at 38°. It will be shown in the next paper (Slater, 1950) that Co I H₂ and succinate reduce added cytochrome *c* at about the same rate in the presence of heart-muscle preparation.

The Co I H₂ and succinic oxidase systems together probably account for a considerable proportion of the total respiration of the muscle, since succinate is undoubtedly an important intermediary metabolite and several metabolites require cozymase for their oxidation.

SUMMARY

1. The method of measuring the dihydrocozymase (Co I H₂) oxidase activity of heart-muscle and kidney preparations is described.

2. Co I H₂ rapidly and completely reduced cytochromes *a*, *a*₃ and *c* when added to heart-muscle preparation. Cytochrome *b* was slowly and usually incompletely reduced. It was reoxidized on the subsequent addition of fumarate.

3. The Co I H₂ oxidase system was inhibited by

cyanide, sulphide, azide, hydroxylamine and carbon monoxide. The inhibition by cyanide was completely reversed by dilution and that by carbon monoxide completely reversed by strong light.

4. The oxidase system was also inhibited to the extent of 99% by treatment of the heart-muscle preparation with BAL (2:3-dimercaptopropanol) in air. The Co I H₂ oxidase system was rather more susceptible than the succinic oxidase system to low concentrations of BAL. The degree of inactivation caused by BAL was not affected by the addition of diaphorase or of cytochrome *c* after the BAL treatment.

5. Added cytochrome *c* had only a slight effect on the Co I H₂ oxidase system in heart-muscle preparation, but increased the activity of kidney preparation by 11-fold.

6. When the aerobic oxidation of Co I H₂ was prevented either by removing oxygen or by adding cyanide, the Co I H₂ was completely oxidized by fumarate. This anaerobic oxidation was only one-fiftieth as rapid as the aerobic oxidation of Co I H₂ or succinate. It was inhibited by malonate and, to the extent of 60%, by treatment of the heart-muscle preparation with BAL.

7. The diaphorase activity of heart-muscle preparation (measured by extrapolation to infinite methylene blue concentration) was 3–4 times that of the Co I H₂ oxidase system (measured in the absence of added cytochrome *c*).

8. The Co I H₂ oxidase activities of heart-muscle and kidney preparations are about the same as the succinic oxidase activities at room temperature. Since, however, the temperature coefficient of the former is much higher than that of the latter, the Co I H₂ oxidase system is appreciably the more active at 38°.

9. It is suggested that the components of the Co I H₂ oxidase system are diaphorase, the BAL-sensitive factor, cytochrome *c*, cytochrome *a* and cytochrome *a*₃.

I wish to thank Prof. D. Keilin, F.R.S., for his interest in and advice during this investigation and the Australian National University for a Research Fellowship.

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The Dihydrocozymase-cytochrome *c* Reductase Activity of Heart-muscle Preparation

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(Received 10 November 1949)

The dihydrocozymase-cytochrome *c* reductase may be defined, following Potter & Albaum (1943), as that portion of the Co I H₂-oxidase system which is concerned with the reduction of cytochrome *c* by Co I H₂. According to the scheme proposed in the previous paper, it may be represented thus

This paper is concerned with some properties of this enzyme complex in Keilin & Hartree's (1947) heart-muscle preparation. No attempt has been made to obtain it in solution and purify it.

METHODS

Cytochrome c reductase activity was measured by following the reduction of cytochrome *c* in a Beckman spectrophotometer at 550 mμ., using 1 cm. cells. The reoxidation of the cytochrome *c* was prevented by adding cyanide (0.001 M). The diluted heart-muscle preparation was added to cytochrome *c* in phosphate buffer, pH 7.3 (final concentration 0.125 M) in the Beckman cell several minutes before zero

time in order completely to oxidize the cytochrome *c*. At zero time, the cyanide and Co I H₂ solutions were added and the optical density at 550 mμ. measured at approximately 20 sec. intervals. The rates of reduction of cytochrome *c* were sufficiently rapid so that only a small amount of oxidized cytochrome *c* combined with cyanide during the course of the reaction (Potter, 1941; Horecker & Kornberg,

1946). The 'blank' cuvette contained cytochrome *c*, heart-muscle preparation, cyanide and phosphate buffer. Corrections for temperature were made as described in the previous paper (Slater, 1950).

Optical densities at 550 mμ. were converted to molar concentrations of reduced cytochrome *c* by multiplying by the factor 5.35×10^{-5} . This factor was calculated from the molar extinction coefficient at 550 mμ. of reduced cytochrome *c*, reported by Theorell & Akesson (1941), and the observation that, under the conditions of the experiments described in this paper, the optical density of oxidized cytochrome *c* at 550 mμ. was 0.34 times that of reduced cytochrome *c*.

Other measurements were made as described in the previous paper (Slater, 1950). The Co I H₂ was prepared by enzymic reduction. Optical densities at 340 mμ. were con-