A Comparative Study of the Succinic Dehydrogenase-Cytochrome System in Heart Muscle and in Kidney

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Most of our knowledge concerning the components of the system of enzymes which catalyses the aerobic oxidation of succinate has been gained from studies of this system in enzyme preparations obtained from heart muscle. Since, in recent years, kidney has been used by many workers as the source of the succinic oxidase system, it seemed desirable to make a comparative study of this enzyme system in kidney and heart muscle. Such a comparison seemed especially necessary in view of the statement of Keilin & Hartree (1940) that kidney (as well as liver and other organs) does not contain a normal cytochrome *b*, which is an essential component of the succinic oxidase system in heart muscle.

This paper describes a study of the succinic oxidase system in the two tissues, using both manometric and spectroscopic methods. A preliminary account of some of these findings has appeared elsewhere (Slater, 1948).

METHODS

Enzyme preparations

Heart-muscle preparation. This was prepared from horse heart according to the method of Keilin & Hartree (1947). Minced heart (330 g.) was thoroughly washed by stirring with about 5 l. tap water for about 15 min. The mince was collected on muslin, squeezed hard to remove water, and this process repeated about 8 times until the wash liquor was colourless. The washed muscle was then ground in a mechanical mortar with 100 g. sand (acid washed) and 500 ml. 0·02 M-phosphate buffer, pH 7·3, for 2 hr. The thick suspension was diluted with 200 ml. 0·02 M-phosphate buffer and centrifuged for 20 min. at 2000 rev./min. The supernatant cloudy solution was cooled to 0–5° and brought to pH 5·7 with N-acetic acid. The precipitate was immediately collected by centrifuging in the cold at 2000 rev./min. for 15 min., the supernatant discarded, and the residue suspended in an equal volume of 0·1 M-phosphate buffer, pH 7·3. The pH of this preparation was 7·1.

The variation in the activity of different preparations was quite small; the Q_{O_2} of the succinic oxidase system for twenty-one preparations varied between 307 and 740, but was usually between 550 and 700, when fresh heart was used.

Keilin & Hartree (1938) introduced the step involving precipitation with acid for two reasons, viz. (1) to concentrate the preparation in order to make it more suitable for spectroscopic purposes, and (2) to remove the last traces of soluble substances such as cozymase, which inhibits the oxidation of succinate by heart-muscle preparation (Keilin & Hartree, 1940), and myoglobin, which interferes with the spectroscopic observations of the cytochromes. In the present study it was found that the precipitation with acid increased the enzymic activity of the original phosphate extract by 50%, probably due to the removal of proteins which are soluble at pH 5·7, as well as to removal of inhibitors. Activities about 20% higher than those obtained by the Keilin & Hartree (1947) method were obtained by centrifuging the neutral phosphate extract at high speed (12,000 rev./min.), instead of acidifying. The activities of the succinic dehydrogenase, the cytochrome oxidase and of the complete succinic oxidase system were all increased to about the same degree. Probably this difference between the activities obtained by the two methods is due to protein which is soluble at pH 7·3, but precipitated at pH 5·7. The amount of this protein will depend, to a certain extent, on the thoroughness of the preliminary washing of the heart-muscle mince.

The succinic oxidase system is distinctly unstable at pH 5·7 at room temperature, and it is important to carry out the precipitation and subsequent centrifugation at below 5°. Since the succinic oxidase system is much more unstable to acid than the succinic dehydrogenase or the cytochrome oxidase (Keilin & Hartree, 1940), the finding that the relative activities of the succinic oxidase system in the two preparations, obtained by high-speed centrifugation and acidification respectively, are approximately the same as the relative activities of succinic dehydrogenase and cytochrome oxidase shows that acidification at low temperature does not cause any inactivation of the succinic oxidase system. It was found that the heart-muscle preparation could be precipitated a second time at pH 5·7 at 5°, washed with cold water and resuspended in phosphate buffer without loss of activity, but a further precipitation caused a considerable loss of activity. This is probably due to a physical effect on the macromolecular particles, since the inactivation can be reversed by treatment with various protein preparations (Stern & Melnick, 1939; Keilin & Hartree, 1949). Precipitation at pH 4·6 at room temperature, as used by some authors (e.g. Ogston & Green, 1935), is certain to lead to considerable inactivation.

Kidney preparation. Pieces of horse-kidney cortex about $1 \times 1 \times \frac{1}{4}$ in. were treated in a Waring blender with 0·02 M-phosphate buffer for about 5 min. The mixture was centrifuged for 20 min. at 2000 rev./min., and the cloudy red supernatant centrifuged at 12,000 rev./min. for 30 min. in a Servall angle centrifuge. The supernatant was discarded and the residue washed twice with water, centrifuging for 30 min. at 12,000 rev./min. after each washing. The residue was suspended in an equal volume of 0·1 M-phosphate buffer, pH 7·3.

Both the heart-muscle and kidney preparations used in the present investigation contained a considerable amount

of fat, viz. 30% in the heart-muscle preparation (cf. Stern, 1939) and 20% in the kidney preparation.

Cytochrome c. The cytochrome *c*, which was prepared by the method of Keilin & Hartree (1945), contained 0·34% Fe. The concentration of cytochrome *c* in the solution was determined spectrophotometrically, using a Hilger-Nutting spectrophotometer.

Measurement of enzyme activities

All measurements of enzyme activities were made in Barcroft differential manometers at 37–39°. Phosphate buffer, pH 7·3, was used in all methods. All activities are expressed as Q_{O_2} (μl. O_2/mg. fat-free dry wt./hr.).

Succinic oxidase system. The activity of the complete succinic oxidase system was measured in the presence of excess cytochrome *c*. To 0·2 ml. of a heart-muscle preparation, diluted fivefold with 0·18M-phosphate buffer, were added 2·7 ml. 0·18M-phosphate buffer and 0·2 ml. 1·1% cytochrome *c*; after temperature equilibration, 0·2 ml. 0·4M-Na succinate was added by dislodging a dangling tube. The equilibration was carried out for not longer than 20 min. and at a low rate of shaking, since rapid shaking for long periods caused considerable inactivation of the succinic oxidase system (20% after 1 hr.) and of cytochrome oxidase, with little effect on the succinic dehydrogenase. The activity of the kidney preparation was measured in the same way, except that 1·7 ml. of the phosphate buffer and 1·0 ml. water were used. Final concentrations were: phosphate, 0·15M in the case of the heart-muscle preparation and 0·10M in the case of the kidney preparation; succinate, 0·024M; cytochrome *c*, 4×10^{-5}M. The O_2 uptake between 5 and 15 min. after adding the succinate was used as the measure of the activity of the succinic oxidase system. This rate is about 5% less than the initial rate (obtained by extrapolation).

Succinic dehydrogenase. To 0·2 ml. of an enzyme preparation, diluted fivefold with 0·18M-phosphate buffer, were added 2·3 ml. (heart muscle) or 1·7 ml. (kidney) 0·18M-phosphate buffer, 0·3 ml. 0·01M-methylene blue and 0·3 ml. 0·1M-KCN (neutralized). In the case of the kidney preparation, 0·6 ml. water was also added. After temperature equilibration, 0·2 ml. 0·4M-Na succinate was added by dislodging a dangling tube. Final concentrations were phosphate, 0·15M (heart muscle) or 0·10M (kidney); succinate, 0·024M; methylene blue, 0·0009M; cyanide, 0·009M. The O_2 uptake between 5 and 15 min., or between 5 and 25 min., after the addition of succinate was used as the measure of succinic dehydrogenase activity (the rate of O_2 uptake was practically constant for 15 min. after the addition of succinate).

Cytochrome oxidase was determined by the method described in another paper (Slater, 1949*a*).

Determination of concentrations of haematin compounds

Total protohaematin. The total protohaematin content was determined by matching, with a low dispersion microspectroscope, the intensity of the 548–560 mμ. band obtained by adding pyridine and reducing agent with that of the 551–560 mμ. band of pyridine protohaemochromogen, obtained in the same way from pure protohaemin. When pyridine (0·25 vol.) and $Na_2S_2O_4$ were added to the heart-muscle preparation, it turned pink in colour and the bands of cytochrome *c* (548–552 mμ.) and cytochrome *b* (562–566 mμ.) were replaced by a broad band at 548–560 mμ. (cf. Keilin, 1926). It was found that the addition of pyridine and $Na_2S_2O_4$ to cytochrome *c* did not alter its absorption spectrum. (This may be because cytochrome *c*, which is unusually stable, does not form a pyridine haemochromogen under these conditions. In any case, however, the position and probably the intensity of the band would not be altered since Hill & Keilin (1930) found that the haematin of cytochrome *c* gave a pyridine haemochromogen with bands in the same position as cytochrome *c*.) The short wave length margin of the broad band is therefore derived from cytochrome *c*. The longer wave length portion of this band is due to pyridine protohaemochromogen, derived from protohaematin compounds present in the heart-muscle preparation. A mixture of pure cytochrome *c* and pyridine haemochromogen from pure haemin in the same concentrations as found in heart-muscle preparation gave a band at 548–560 mμ. indistinguishable from that given by heart muscle. The value obtained for the total protohaematin content is probably slightly high, since the cytochrome *c* present in the heart-muscle preparation will show some absorption in the middle of the 551–560 mμ. band, but the error is quite small especially in the case of the kidney preparation, since the total protohaematin content of both preparations is much higher than that of cytochrome *c*.

Cytochrome c. The cytochrome *c* content was determined by direct comparison of the intensity of the 548–552 mμ. band obtained by adding $Na_2S_2O_4$ to heart-muscle preparation with the band obtained from pure cytochrome *c*, standardized spectrophotometrically. This value also may be a little high, owing to the probability of absorption of cytochrome *b* at this wave length. However, it appears that this error is not very serious, since treatment of the preparation with ascorbic acid under anaerobic conditions, which causes the appearance of the *c* band only, gave essentially the same results.

Cytochrome b. The figures for the cytochrome *b* content are much less certain than the other values, since pure cytochrome *b* is not available for comparison. The figure for the heart-muscle preparation was calculated in the following way. The intensity of the *b* band was considered to be about 20% less than that of the *c* band; it was assumed* that the absorption coefficient of cytochrome *b* is the same as that of cytochrome *c*, since both are haemochromogens of very similar haems. The cytochrome *b* content of the heart-muscle preparation was therefore 80% of the cytochrome *c* content. In kidney preparation the two bands were of about equal intensity.

Cytochrome $a + a_3$ content. The relative cytochrome $a + a_3$ content was determined by comparing the intensities of the band at 603–607 mμ.; this is mainly due to cytochrome *a*, but the relative concentration of *a* and a_3 appears to be constant (Keilin & Hartree, 1939).

Weight of the fat-free dried enzyme preparation

Enzyme preparation (1 ml.) was diluted with 5 ml. water in a weighed centrifuge tube, and 1 ml. 20% trichloroacetic acid added. The flocculent precipitate was

* The validity of this assumption is supported by the finding of Bach, Dixon & Zerfas (1946) that the absorption coefficient of the α band of cytochrome b_2 is of the same order as that of cytochrome *c*.

collected by centrifugation, the supernatant siphoned off and the residue washed by centrifugation, once with 5 ml. 50% ethanol and once with 5 ml. 96% ethanol. The residue was dried to constant weight at 100°.

RESULTS

Examination of the methods of measuring the activities of succinic dehydrogenase and of the succinic oxidase system

Substrate concentration. The effect of substrate concentration on the activity of the succinic oxidase system and of succinic dehydrogenase in the heart-muscle preparation is shown in Fig. 1. High succinate concentrations cause a definite inhibition of the complete system but not of succinic dehydrogenase. This suggests that the inhibitory effect of high succinate concentrations on the succinic oxidase system previously reported by several investigators is not due to a specific action of succinate, but is a salt effect. Other salts, for example phosphate buffer, have the same action on the complete system, with little effect on the succinic dehydrogenase

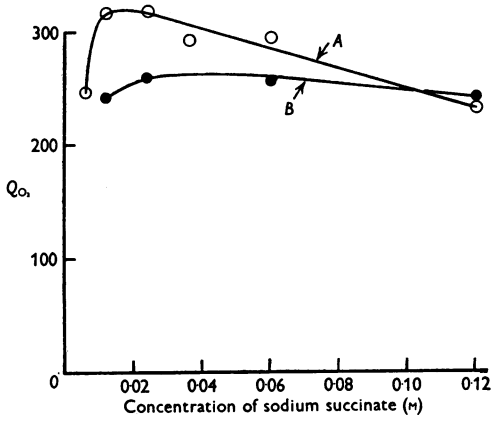

Fig. 1. The effect of concentration of sodium succinate on the activity of the succinic oxidase system (curve *A*) and of succinic dehydrogenase (curve *B*) in heart-muscle preparation (0·04 ml.). Curve *A*, phosphate, 0·15M; cytochrome *c*, 4×10^{-5}M. Curve *B*, phosphate, 0·15M; methylene blue, 0·0009M; cyanide, 0·009M. (Different enzyme preparations were used for the measurements of the succinic oxidase system and of succinic dehydrogenase.)

(Keilin & Hartree, 1949; Slater, 1949*b*). The optimal succinate concentration is about 0·02M for both succinic dehydrogenase and the complete system. This is a little lower than the optimum found by Schneider & Potter (1943), using a different type of preparation (kidney homogenate). The finding of Elliott & Grieg (1938) that the optimal succinate concentration is less for the reaction with methylene blue than with oxygen is not supported by Fig. 1.

Concentration of methylene blue. Methylene blue is a very sluggish carrier compared with the cytochrome system. The activity of the succinic dehydrogenase was increased by increasing the methylene blue concentration throughout the range shown in Fig. 2. However, it was decided to limit

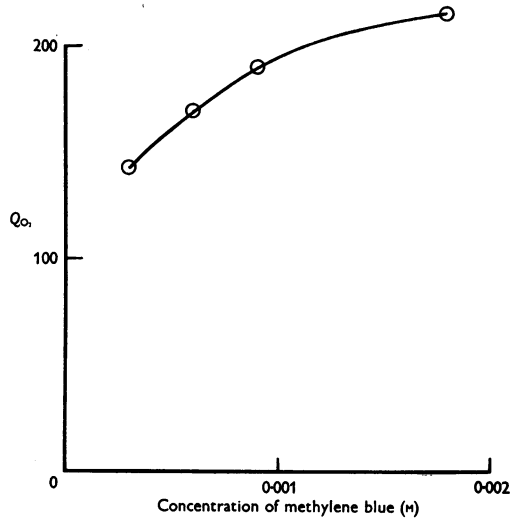

Fig. 2. The effect of concentration of methylene blue on the activity of succinic dehydrogenase in heart-muscle preparation; succinate, 0·024M; cyanide, 0·009M; phosphate, 0·15M; 0·04 ml. heart-muscle preparation.

the methylene blue concentration to 0·0009M, even though this concentration was far below the optimum, to avoid the toxic action of methylene blue on the enzyme. The presence of 0·01M-cyanide, added to prevent oxidation through the cytochrome system, inhibits the rate of reduction of methylene blue by succinate. For these reasons the absolute values of the succinic dehydrogenase activity determined by the method described have no significance and cannot be compared with the activity of the complete system. This is the explanation of the apparent anomaly that the activity of the complete system in a carefully prepared heart-muscle preparation is always greater than that of succinic dehydrogenase, which is an essential constituent of the complete system. The method described is, however, suitable for comparing succinic dehydrogenase activities, if measurements are always carried out in the same way.

Cytochrome c concentration. The effect of the concentration of added cytochrome *c* on the activity of the complete succinic oxidase system in the heart-muscle and kidney preparations is shown in Fig. 3. With both preparations, 4×10^{-5}M added cytochrome *c* was sufficient for practically optimal activity.

Enzyme concentration. The effect of enzyme concentration on the succinic oxidase and succinic dehydrogenase activities is shown in Fig. 4. Under the conditions of the experiments, a straight line, which

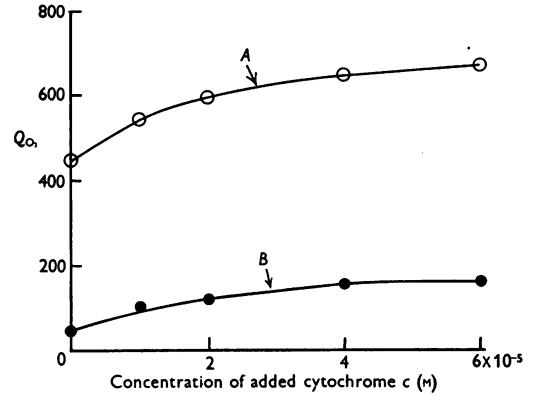

Fig. 3. The effect of concentration of added cytochrome *c* on the activity of the succinic oxidase system in heart-muscle preparation (curve *A*) and kidney preparation (curve *B*); succinate, 0·024M; phosphate, 0·15M (heart muscle) and 0·10M (kidney); 0·04 ml. enzyme preparation.

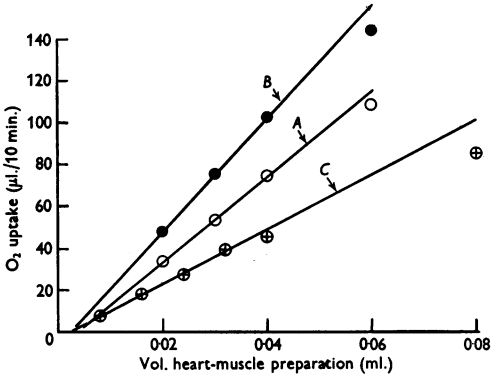

Fig. 4. The effect of concentration of heart-muscle preparation on the rate of oxidation of succinate in phosphate buffer (0·15M) alone (curve *A*), in the presence of 4×10^{-5}M added cytochrome *c* (curve *B*), and in the presence of potassium cyanide (0·009M) and 0·0009M methylene blue (curve *C*). Succinate (in all cases), 0·024M.

did not pass through the origin, was obtained up to a concentration of 0·04 ml. heart-muscle preparation per flask (total fluid volume = 3·3 ml.). There was a slight falling away from this straight line at higher concentrations.

Phosphate concentration. The concentration of phosphate buffer has a marked effect on the activities of the succinic oxidase system (Keilin & Hartree, 1949; Slater, 1949*b*) and of the cytochrome oxidase (Quinlan-Watson & Dewey, 1948; Slater, 1949*a*), but has little influence on the succinic dehydrogenase (Slater, 1949*b*). The concentrations of phosphate buffer given in the above methods are approximately optimal for the enzyme concerned.

Cytochrome system in kidney preparation

Kidney cannot be washed free from haemoglobin in the same way as heart muscle, since minced kidney is readily dispersed merely by washing in water. Accordingly, Keilin & Hartree (1940) used such a dispersion of kidney as the starting material for their preparation, and removed the haemoglobin and other soluble substances by precipitating at pH 5·5. It should be noted that, in this case, the acid precipitation was used to remove all the soluble substances which could be removed in this manner; in the case of the heart muscle, this treatment is used only to remove the last traces of soluble substances which survive the exhaustive preliminary washing of the minced heart muscle. As suitable apparatus was available, it was decided to use a different method from that used by Keilin & Hartree (1940) for preparing the enzyme solution. The kidney was dispersed in a Waring blender, and the resultant strongly coloured dispersion was freed from soluble substances by centrifugation at high speed and washing the residue with water. The method is described in detail on p. 1.

The preparation showed a very faint band at 580 mµ., probably due to traces of residual haemoglobin. On adding succinate, a spectrum essentially the same as that obtained with heart muscle was observed, but with the following differences: (1) all bands, but particularly the *b* and *c*, were considerably weaker (see p. 5); (2) the *b* band was at 560 mµ., compared with 564 mµ. in the case of the heart-muscle preparation. As is the case with heart-muscle preparation, the bands disappeared on shaking with air, but reappeared much more slowly than in heart muscle when the shaking was stopped. This shows that the succinic dehydrogenase activity relative to that of cytochrome oxidase was considerably lower in the kidney preparation, a conclusion which was confirmed by manometric experiments (see p. 5).

When $Na_2S_2O_4$ was used instead of succinate, the *a* band was the same as before, but a strong shading appeared between the *b* and *c* bands, giving the appearance of a single diffuse band with two maxima. Thus the kidney preparation contains a substance which gives with $Na_2S_2O_4$, but not with succinate, a band at about 550–560 mµ.; this may be denatured protein-haemochromogen. At the temperature of liquid air, a kidney preparation diluted fivefold and treated with $Na_2S_2O_4$ showed two absorption bands, a strong band at 555–559 mµ. and a weaker band at 547–549 mµ. It seems that at the temperature of liquid air the band due to cytochrome *c* shifts towards the blue end of the spectrum, while the 550–560 mµ. band is narrowed and intensified and

fuses with that of cytochrome *b*, which also moves towards the short wave length.

Pyridine and $Na_2S_2O_4$ gave a spectrum essentially the same as that obtained with heart-muscle preparation, viz. an intense band at 548–560 mμ. and a weaker band at 580–590 mμ. The band at 548–560 mμ. was as intense as with heart muscle, while the 580–590 mμ. band was much weaker. At the temperature of liquid air, the former band was intensified and sharpened (550–554 mμ.).

Cytochromes *b* and *c* can be readily differentiated by making use of the following properties: (1) quinol, *p*-phenylenediamine and ascorbic acid reduce cytochromes *c* and *a* only, not cytochrome *b* (a very faint *b* band appears with ascorbic acid, but only after standing for about 30 min.); (2) when succinate and phenylurethane are added to the enzyme preparation and the mixture shaken with air, the *b* band alone is visible (cf. Keilin, 1925; Keilin & Hartree, 1940). The heart-muscle and kidney preparations behaved in the same way, as regards these reactions. Thus, kidney contains both cytochromes *b* and *c* which appear to have essentially the same properties as in heart muscle. The only qualitative difference is that the cytochrome *b* band occupies a slightly different position.

Keilin & Hartree (1940) found that, in kidney preparations, the usual cytochrome *b* and *c* bands were replaced by a single wider band (with centre at 555 mμ.), which was considered to be the same as that of cytochrome b_1 found in certain micro-organisms. The present work shows, however, that these observations of Keilin & Hartree (1940) can be explained by the presence of a compound reducible by $Na_2S_2O_4$ which masks the bands of cytochromes *b* and *c*, since these authors used $Na_2S_2O_4$, not sodium succinate, as the reducing agent. If, as is probable, this compound is denatured protein haemochromogen, it would be expected that Keilin & Hartree's (1940) preparation, obtained by acid precipitation, would contain more of this compound than the preparation used in the present investigation; consequently, the two maxima which were observed even with $Na_2S_2O_4$ at room temperature in the present study might be completely masked and only one band be visible. The spectrum obtained with $Na_2S_2O_4$ at the temperature of liquid air was approximately the same as that described by Keilin & Hartree (1940), except that the latter authors give the position of the weaker band as 551 mμ., compared with 548 mμ. obtained in the present study. This difference could be explained by the relatively greater intensity of the stronger band in the Keilin & Hartree (1940) preparation compared with that in the preparation used in the present study, since the position of a weak band can be changed by a strong band in the near vicinity. This weakens Keilin & Hartree's argument that, since at the temperature of liquid air the band of pure cytochrome *c* shifts to 547 mμ., the kidney preparation contains no cytochrome *c*.

Comparison of the succinic dehydrogenase-cytochrome system in heart and kidney preparations

In Table 1 are compared the activities in heart-muscle and kidney preparations of the complete succinic oxidase system and of its component parts, the succinic dehydrogenase and cytochrome oxidase,

Table 1. *Relative enzyme activities and concentrations of haematin compounds in heart-muscle and kidney preparations*

(All activities are based on fat-free dry weight. The methods of determining succinic oxidase, succinic dehydrogenase and cytochrome oxidase activities and the amounts of cytochrome *c*, cytochrome *b*, cytochrome $a+a_3$ and total protohaematin compounds are described under 'Methods'. The amount of catalase was determined by comparing the rate of evolution of O_2 when H_2O_2 was added to a suitably diluted heart-muscle or kidney preparation with that obtained, under the same conditions, by adding H_2O_2 to the same enzyme preparation to which a small amount of a solution of pure catalase was added. The concentration of catalase in the latter solution was determined by comparing the intensity of the pyridine haemochromogen band with that obtained from crystalline haemin.)

	Heart muscle	Kidney	Ratio, heart-muscle: kidney
Succinic oxidase (Q_{O_2})	625	200	3·1
Succinic dehydrogenase (Q_{O_2})	240	67	3·6
Cytochrome oxidase (Q_{O_2})	3200	1380	2·3
Protohaematin (μmol./g.)	1·8	1·8	1·0
Cytochrome *c* (μmol./g.)	0·8	0·27	3·0
Cytochrome *b* (μmol. haematin/g.)	0·64	0·27	2·4
Catalase (μmol. haematin/g.)	0·002	0·034	0·05
Cytochrome $a+a_3$ (arbitrary units)	1	0·54	1·9

and also of the amounts of the cytochromes and protohaematin compounds. It should be noted that the actual figures for the succinic dehydrogenase activity have no absolute significance, and can be compared only with one another, not with the succinic oxidase and cytochrome oxidase activities. The cytochrome oxidase activity can, however, be compared with the activity of the complete succinic oxidase system. The activities of the succinic dehydrogenase and of the complete succinic oxidase system are about three times as high in the heart-muscle as in the kidney preparation, and this ratio is similar to the relative intensities of the cytochrome *b* band. This agrees with the usual finding that cytochrome *b* is closely associated with succinic dehydrogenase. The heart-muscle preparation has about twice the cytochrome oxidase activity of the kidney preparation and also about twice the content of cytochrome $a+a_3$, which agrees with the finding of Keilin & Hartree (1939) that there is a fairly close correlation between the cytochrome oxidase activity and the intensity of the $a+a_3$ band.

It has been mentioned above that the figure given for the protohaematin content does not include more than a small fraction of the cytochrome *c*. It does probably include the cytochrome *b*, but the amount of this component is quite insufficient to account for the intensity of the pyridine haemochromogen band. Thus, the total protohaematin content of the heart-muscle preparation is nearly three times that of cytochrome *b*, while the corresponding ratio for the kidney preparation is nearly seven. It is interesting that, although the heart-muscle preparation is much richer than the kidney in the cytochromes, both contain about the same concentration of protohaematin compounds. These figures show that both enzyme preparations contain considerable amounts of a haematin compound (or compounds), whose spectrum is not visible either before or after the addition of $Na_2S_2O_4$. One such compound is catalase, but the amount of this enzyme is insufficient to account for more than a very small fraction of the discrepancy between the cytochrome *b* and the total protohaematin content. The kidney preparation contains traces of haemoglobin and some denatured haematin compounds, but insufficient in amount to account for the sixfold discrepancy in that preparation. Certainly, such compounds are not responsible for the discrepancy in the heart-muscle preparation, since a carefully made heart-muscle preparation shows no signs of haemoglobin or denatured protein compounds.

It is concluded, therefore, that these preparations contain unidentified haematin compounds (or one compound). The fact that the spectra of these compounds are not usually visible in the concentrations in which they are normally found in these preparations suggests that they are not haemochromogens like cytochrome, but resemble compounds of the type of methaemoglobin, catalase or peroxidase, which show only weak absorption bands in either the oxidized or reduced states. That tissues contain a haematin compound in addition to the cytochromes (or even in its absence) was deduced by Keilin (1926, 1929) many years ago on the basis of the great increase in the intensity of the absorption band on the addition of pyridine and reducing agent to many tissues. At first, Keilin believed that the compound was free haematin itself, but he and Hartree (Keilin & Hartree, 1947) have recently shown that haematin is a strong inhibitor of succinic dehydrogenase in low concentrations and have concluded that 'not only the cytochrome compounds, catalase and peroxidase, but all other forms of intracellular haematin exist as compounds with proteins' (see also Keilin & Hartree, 1949). The nature of these haematin compounds must await further investigation. There is evidence (Slater, 1949*c*) that at least a part of this haematin fraction is probably concerned, like the cytochromes, in the transfer of electrons from substrate to molecular oxygen.

The point has been made above that the cytochrome system of kidney preparation does not differ in any important respect from that of the heart-muscle preparation. Similarly, it seems that there are only minor differences in the succinic oxidase systems. It is shown in another paper (Slater, 1949*c*), that both tissues require a factor for the reduction of cytochrome *c* by cytochrome *b*. One point of difference between heart-muscle and kidney preparations is that the endogenous cytochrome *c* of the kidney preparation is relatively less active than that in the heart-muscle preparation. Thus, Fig. 3 shows that the addition of 6×10^{-5} M-cytochrome *c* to the heart-muscle preparation increased the activity by about 50 % (this is a greater increase than found with most heart-muscle preparations; the usual increase is only about 20–30 %), while the same amount of cytochrome *c* had a much greater effect on the kidney preparation, whose activity was increased about 300 %. It is important to note that the concentration of cytochrome *c* necessary for maximum activity (4×10^{-5} M) is very much higher than that actually present in the heart-muscle preparation. In fact, the amount of cytochrome *c* added to the manometric flask to give this concentration was 3·3 mg., while the total weight of the heart-muscle preparation was usually only 1·2 mg. This illustrates the point already made by Keilin (1930) and Keilin & Hartree (1945, 1949) that added cytochrome *c* is very much less effective catalytically than the cytochrome *c* present in the heart-muscle preparation, where it is presumably attached to the particles in such a way that it is more readily accessible to the remainder of the system. This question is also discussed in another paper (Slater, 1949*a*).

Succinic dehydrogenase and cytochrome b

The suggestion has been made by Bach *et al.* (1946) and by Ball, Anfinsen & Cooper (1947) that cytochrome *b* and succinic dehydrogenase are identical. To avoid confusion, it is necessary to distinguish between two meanings which are given to the term 'succinic dehydrogenase'. Strictly speaking, succinic dehydrogenase means the enzyme which is specifically concerned in the activation of succinate; however, it is often used to refer to that part of the succinic system which is concerned in the catalysis of the reduction of methylene blue by succinate. The following experiment was carried out to test the possibility that cytochrome *b* is part of succinic dehydrogenase, in this latter sense.

A heart-muscle preparation (2 ml.) was placed in the main compartment of a modified Thunberg tube, with a side arm (described by Keilin & Hartree, 1947); 0·1 ml. 0·003 M-sodium succinate was placed in the side arm, and 0·3 ml. 0·001 M-methylene blue

in the hollow stopper. After evacuation, the succinate was added to the heart muscle and the spectrum observed with a low dispersion microspectroscope. The spectrum was the same as that obtained with a higher concentration of succinate except that the *b* band was a little weaker. On adding the methylene blue, the *b* band immediately almost completely disappeared while the *c* and $a+a_3$ bands remained visible. The methylene blue was only partially reduced, but its spectrum did not seriously obscure that of the cytochromes. When 2:6-dichlorophenolindophenol was used instead of methylene blue, the dye was immediately decolorized and the *b* band immediately disappeared. These observations strongly suggest that cytochrome *b* is involved in the reduction of methylene blue and 2:6-dichlorophenolindophenol by succinate. It is impossible at present to state whether cytochrome *b* is directly reduced by succinate or if this reduction requires an additional enzyme, which is the true succinic dehydrogenase. The solution of this question must await the isolation of either succinic dehydrogenase or cytochrome *b*, which can then be examined to see if it has the properties of the other.

DISCUSSION

Both the heart-muscle and kidney preparations are largely composed of small particles in colloidal solution, possessing a very high enzymic activity. Keilin & Hartree (1938, 1939, 1940) have pointed out that such preparations behave in many respects like the living cell: in particular, the respiration is affected by inhibitors in the same way as is the respiration of the living cell. These preparations, especially the more active one from heart muscle, are therefore very suitable for the study of many problems concerned with intracellular respiratory catalysis. The absence of a cell wall eliminates difficulties associated with diffusion through such a barrier, but it has been suggested (Slater, 1949*a*) that problems of diffusion, especially of large molecules, cannot always be dismissed when working with such a preparation. Keilin & Hartree (1949) have shown that the physical properties of these preparations are of paramount importance and must be carefully controlled in studies of the action of inhibitors. This point is further examined in another paper (Slater, 1949*b*).

It might be expected that the drastic mechanical treatment given, especially to the heart muscle, viz. grinding with sand in a mortar for 2 hr., would completely destroy the organization which exists within the living cell. However, this cannot be the case, since the particles have a very high enzymic activity, even of the complex succinic oxidase system (the activity of which is, in fact, much higher in the heart-muscle preparation than in the washed minced heart muscle). An active succinic oxidase system must depend on a considerable degree of organization, with each component situated in the correct spatial relationship to the component with which it reacts. It seems preferable, then, to regard these enzyme preparations as being solutions of some subcellular structure or macromolecules, rather than of unspecific fragments of the mass of the protoplasm.

Keilin & Hartree (1939) have pointed out that the heart-muscle preparation contains a considerable amount of copper. This was confirmed in the present investigation. The total copper content of the heart-muscle preparation was found to be 0·0123 %, a figure very similar to the 0·0129 % of non-dialysable copper found by Keilin & Hartree (1939). This is equivalent to 1·9 micromol. of copper/g. of fat-free heart-muscle preparation, which is the same order of magnitude as the total protohaematin content. It seems not unlikely that this copper is in the form of some catalytically active protein compound.

The Q_{O_2}, at 37°, of the cytochrome *c* in the heart-muscle preparation may be calculated from the figures in Table 1. For purposes of this calculation, it should be noted that the value for the succinic oxidase activity in Table 1 was obtained by measuring the rate of oxidation of succinate in the presence of excess cytochrome *c*. In the absence of added cytochrome *c* the value is about 20 % lower; from this figure the Q_{O_2} of cytochrome *c* at 37° in the heart-muscle preparation is 38,000. This is about half the value found by Keilin & Hartree (1940) for yeast, viz. 80,000. The discrepancy is not surprising, since one would hardly expect to have retained, in the heart-muscle preparation, the complete organization existing in the living cell. That the factor is as low as 2 indicates that a high degree of organization is retained.

The activity of the cytochrome oxidase is five times that of the succinic oxidase system. This is not unexpected since, in the cell, the oxidation of many substrates, in addition to succinate, passes through the cytochrome oxidase system.

SUMMARY

1. Methods of obtaining, from heart muscle and kidney, enzyme preparations which are suitable for the study of the succinic oxidase system and for the spectroscopic study of the cytochromes are described.

2. The factors involved in the measurement of the succinic dehydrogenase activity and of the complete succinic oxidase system have been investigated.

3. Kidney possesses essentially the same cytochrome system as is found in heart muscle. There is no evidence that cytochrome b_1 replaces cytochromes *b* and *c* in the kidney.

4. Quantitative measurements of the amounts of haematin compounds in the enzyme preparations suggest that both contain unknown haematin compounds, whose spectra are not normally visible.

5. Evidence is produced suggesting that cytochrome *b* is involved in the catalysis of the reduction of methylene blue by succinate.

6. The enzyme preparations are colloidal solutions of particles, which are probably derived from some subcellular structure in the tissue.

7. The fact that the molar concentration of copper in the heart-muscle preparation is of the same order of magnitude as that of the haematin compounds suggests that a copper-protein compound might have some catalytic function in the preparation.

8. The Q_{O_2} (at 37°) of the cytochrome *c* in the heart-muscle preparation is 38,000, i.e. about half the value in yeast.

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The Action of Inhibitors on the System of Enzymes which Catalyse the Aerobic Oxidation of Succinate

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As has been discussed in the previous paper (Slater, 1949*a*), the enzyme preparations used in the investigation of the succinic oxidase system are colloidal solutions of particles, which are probably derived from some subcellular structure in the tissue. The oxidation of succinate by molecular oxygen requires the co-operation of a number of electron or hydrogen carriers, which appear to be firmly attached to these particles. It is obvious that any one carrier will be unable to react with the carriers before and after it in the reaction chain unless the three are closely situated in or on the particle. Thus, the maintenance of the structure of the particle, which keeps these carriers in the correct spatial relationship to one another, is of paramount importance for the activity of the succinic oxidase system, as has been pointed out by Keilin & Hartree (1940, 1949).

Substances which combine with specific groups in the enzyme molecule, thereby inhibiting the reaction catalysed by the enzyme, are widely used for studying the properties of enzymes. When dealing with a complex system of the type of the succinic oxidase system, the possibility must be considered that the inhibitor does not combine with any specific groups on the enzyme molecule, but acts non-specifically on the enzyme system as a whole, by affecting the properties of the particles in the solution in such a way as to impair the mutual accessibility of the components of the system. Thus, before inhibitors can profitably be used to obtain information about the components of the succinic oxidase system, the characteristics of the action of inhibitors of this latter type must be determined.

METHODS

The methods of obtaining the enzyme preparations and of measuring enzymic activities have already been described (Slater, 1949*a*). Except where otherwise stated, the succinic oxidase activity was measured in the presence of added cytochrome *c* (4×10^{-5} M). All activities are expressed as Q_{O_2} (μl. O_2/mg. fat-free dry wt./hr.). Most of the measurements of succinic dehydrogenase activity were made by the manometric method used in the previous paper, but in some cases the Thunberg procedure was used.

Denatured globin (kindly supplied by Dr E. F. Hartree) had been prepared by the method of Keilin & Hartree (1947).

$Ca_3(PO_4)_2$ gel was prepared according to the method of Keilin & Hartree (1938).