that the transition to the steady rate cannot be due to any partial destruction of the enzyme. If it were, no increase in O_2 evolution would be observed on dilution. The explanation for this transition from α - to β -activity in the decomposition of H_2O_2 by catalase should be sought in the series of unit reactions involved in the peroxide decomposition. In this respect there is a similarity in the kinetics of the enzymatic decomposition and of the reaction catalyzed by ferrous iron (George, 1947), even though the iron in catalase is in the ferric state.

SUMMARY

1. The evolution of oxygen from hydrogen peroxide at pH 5.58 by liver catalase, erythrocyte catalase and a suspension of plasmolyzed red blood corpuscles has been measured manometrically, and by the 'boat technique' of Meldrum & Roughton (1934) using a pressure gauge for following rapid rates. There is an initial rapid evolution of O_2 followed by a slower steady evolution: a transition from α - to β -activity.

2. Identical kinetic relationships are obtained with the three specimens of catalase. The rate is directly proportional to the catalase concentration. The variation of the rate with peroxide concentration shows a maximum at about 0.07 M- H_2O_2 , further increase in the H_2O_2 concentration decreasing the rate very markedly.

3. These kinetic relationships, particularly the inhibition at high H_2O_2 concentrations, are in no way determined by the purity of the enzyme, the presence of inhibitors in the peroxide or the nature and concentration of the buffer solution used.

4. The quantitative reversal of the inhibition shown by addition of dilute buffer solution or peroxide during the reaction shows that enzyme destruction plays no part in determining these kinetic relationships.

5. Dilution during the period of initial rapid activity shows a similar reversibility which suggests that the transition from α - to β -activity is not caused by the partial destruction of the enzyme.

I wish to thank Prof. D. Keilin, F.R.S., for his stimulating interest and kind encouragement in this work.

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Activity of the Succinic Dehydrogenase-cytochrome System in Different Tissue Preparations

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(Received 25 June 1948)

The object of this paper is the study of the effect of different factors on the rate of aerobic oxidation of succinic acid by various tissue preparations. From the results obtained it is apparent that there are broadly speaking two types of factor: first, those which may react directly and specifically with one or more components of the succinic dehydrogenase-cytochrome system or of other catalytic mechanisms linked to this system; and secondly those which, by modifying the colloidal state of the enzyme prepara-

tions, may markedly increase or decrease their activity. The significance of the latter type of factor has not hitherto been fully recognized, with the result that the mode of action of a number of inhibitors and activators on the succinic system in tissue extracts has been misinterpreted, and the activators have erroneously been considered as essential links in the catalytic chain which brings about the aerobic oxidation of succinate. This study will enable us to examine critically the claims of

previous workers to have discovered or even isolated such additional catalytic intermediates in the succinic system.

MATERIALS AND METHODS

The preparation of heart muscle (from horse unless otherwise stated) and pigeon-breast-muscle extracts and of denatured globin (from recrystallized horse oxyhaemoglobin) have already been described (Keilin & Hartree, 1947*a, b*). If prepared exactly according to our description the heart preparation will yield, on 20-fold dilution with 0.1M-phosphate (pH 7.3), a homogeneous opalescent fluid which shows no sign of aggregation on standing for several hours at room temperature. The appearance of a flocculent precipitate indicates either that the pH became too low (<5.5) during the isolation of the enzyme material, or that it was not kept cold at that stage. Although such materials may show catalytic activity, they are unsuitable for the experiments described in this paper. Cytochrome *c* was prepared from horse heart (Keilin & Hartree, 1945) and a 1% solution of the pigment, containing 0.34% Fe, was generally used. In certain experiments cytochrome *c* containing 0.43% Fe was used.

Kidney oxidase preparations. The cortical layer of 1 horse kidney was passed through a Latapie mincer, stirred 10 min. with 1 l. distilled water and pressed out in muslin. The pulp was stirred with a further 1 l. water and pressed out again. The combined fluids were centrifuged to remove solid particles and the cloudy fluid cooled to 0° was brought to pH 5.5 with *n*-acetic acid. The precipitate was immediately centrifuged down, washed by centrifuging with 0.02M-KH₂PO₄, and the solid material resuspended in its own volume of 0.1M-phosphate buffer (pH 7.3).

Vacuum-dried preparations. Heart-muscle preparation (20 ml.) in a 500 ml. beaker was evacuated to 0.1 mm. Hg in a desiccator over KOH and concentrated H₂SO₄. Within 10–20 min. the material froze as a result of the rapid evaporation. The desiccator was then detached from the pump and allowed to stand evacuated overnight. In this way a light flaky product was obtained, very similar in appearance to a freeze-dried protein.

Frozen oxidase preparations. Heart-muscle preparations were frozen three times, either in liquid air or in an ice-salt freezing mixture, with complete thawing after each freezing. This treatment resulted in an irreversible flocculation of the colloidal suspension.

Glyoxaline buffer. As veronal inhibits succinic dehydrogenase, phosphate-free buffers (pH 7.3) were made up from 4(5)-methylglyoxaline (Windaus & Knoop, 1905) according to the description of Kirby & Neuberger (1938).

The oxidation of succinate was followed in differential manometers at 39° with 3.3 ml. fluid in each flask as already described (Keilin & Hartree, 1947*b*). Unless otherwise stated, the concentration of buffer was 0.07M with respect to phosphate or glyoxaline. The right-hand flasks received the appropriate oxidase preparation and 0.2 ml. 1% cytochrome *c*. Sodium succinate was added from a dangling tube, its concentration after mixing with the contents of the flask being 0.04M. All experiments were carried out at pH 7.3. When frozen or dried preparations were tested this fact is stated; in all other cases the fresh material was used. The term Q_{O_2} used in this paper corresponds to $\mu\text{l. O}_2$ taken up/mg. dry wt. of preparation/hr.

EXPERIMENTAL

A. Effect of varying concentrations of phosphate and glyoxaline buffers

Our experiments have shown that phosphate buffer cannot be considered as an indifferent medium for the study of the succinic dehydrogenase-cytochrome system.

Fig. 1, which summarizes the experimental results obtained with the heart-muscle preparation before and after vacuum drying, clearly indicates that the rate of oxidation of succinic acid increases with the decrease in phosphate concentration down to 0.15M, but at lower concentrations the rate falls again. In the case of the undried material these changes in the activity of the preparation follow the

Fig. 1. Effect of phosphate buffer concentration on the rate of O_2 uptake of fresh and vacuum-dried heart preparations in presence of succinate and cytochrome *c*. Dry wt. of preparation = 1.1 mg.; temp., 39°; pH, 7.3.

degree of dispersion of the protein, the most complete dispersion being at 0.15M-phosphate with a tendency towards flocculation as the concentration of phosphate is either decreased or increased. When phosphate is replaced by the glyoxaline buffer the rate of oxidation of succinate increases with the decrease of buffer concentration. For such experiments glyoxaline buffer must be used instead of phosphate in the preparation of the heart-muscle extract. The peculiar effect of phosphate can be illustrated from experiments where the total salt concentration (phosphate + chloride) remained constant while the phosphate concentration, denoted as [phosph], was varied. In such a case there is a definite activation by phosphate when comparison is made with control experiments in glyoxaline buffer (Table 1).^{*} The combination of this activation by [phosph] and the general salt effect presumably gives rise to the maxima in Fig. 1. No optimal effect of [phosph] is shown by kidney preparations where the reaction rate falls off with increasing

^{*} Throughout this paper we use the term 'activation' to indicate an increase in the rate of O_2 uptake of the entire catalytic system.

Table 1. *Comparison of the effects of phosphate and glyoxaline buffers on the oxidation of succinate by a heart oxidase preparation*

(Total volume of oxidase (0.05 ml.) + cytochrome c + succinate = 1.0 ml. NaCl and buffers: 0.1 M; temperature, 39°; pH, 7.3.)

	O ₂ uptake (μ l./20 min.)	
	In phosphate	In glyoxaline
Oxid. + cyt. c + succ. + 1.3 ml. buffer + 1.0 ml. NaCl	87	97
Oxid. + cyt. c + succ. + 1.8 ml. buffer + 0.5 ml. NaCl	116	100
Oxid. + cyt. c + succ. + 2.3 ml. buffer + 0 ml. NaCl	138	99

Table 2. *The effect of phosphate concentration on the oxidation of succinate by a kidney preparation, and the effect of cytochrome c*

(Kidney oxidase (0.25 ml.); succinate; temp., 39°; pH, 7.3.)

Phosphate conc. (M)	μ l. O ₂ uptake in 10 min.	
	No cyt. c	With cyt. c
0.015	20	96
0.06	14	77
0.15	8	18
0.30	1	9

[phosph] (Table 2). The rate of reduction of methylene blue in Thunberg vacuum tubes by succinate and the various preparations is independent of [phosph] over a wide range.

B. Effect of adenosinetriphosphate (ATP)

Biró & Szent-Györgyi jun. (1946) tested the effect of ATP on the oxidation of succinic acid by a preparation obtained from rabbit-skeletal muscle minced and washed twice in 20 volumes of water. The maximum Q_{O_2} of this muscle preparation at 37° without and with ATP was found by them to be 2.5 and 5 respectively. They concluded that 'the activity of the succinoxidase is greatly dependent on the presence of ATP'.

As the activity of their enzyme system was very low we considered it interesting to determine the effect of ATP on our enzyme preparations, the Q_{O_2} of which varies between 500 and 700. The experiments, carried out in the usual way, showed that the Q_{O_2} of a horse-heart preparation in phosphate buffer without and with 0.003 M-ATP (5 mg./manometer flask) was 620 and 540 respectively. In other words, ATP produced about 13% inhibition. Experiments carried out on a heart-muscle extract prepared and tested in glyoxaline buffer gave similar results: namely, ATP, in presence or in absence of K ions, invariably produced about 15% inhibition. The glyoxaline heart-muscle preparation was found to contain only a trace of inorganic phosphate and its adenosinetriphosphatase (ATP-ase) activity was very low. Thus 0.2 ml. of this preparation (i.e. 4 times the

amount used in manometric experiments) contained 5 μ g. inorganic P and when incubated with 5 mg. ATP at 39° for 50 min., hydrolyzed only 8% of the substrate.

Since the heart-muscle oxidase produced by our method contains very little ATP-ase further experiments were carried out on a pigeon-breast-muscle preparation (Keilin & Hartree, 1947b) using both phosphate and glyoxaline buffers for extraction. Several samples were collected in the course of fractionation of the muscle and these differed in their activities with respect to succinate, and in their contents of myosin and ATP-ase. An examination of these fractions in glyoxaline and in phosphate buffer showed that the addition of ATP produced inhibitions varying from 25 to 37%.

We can say in conclusion that the results of our experiments, unlike those of Biró & Szent-Györgyi jun. (1946), show that ATP inhibits the catalytic activity of highly active preparations oxidizing succinic acid. The inhibitory effect of ATP is, in fact, of the same order as that produced by an equivalent concentration of inorganic pyrophosphate.

C. Effect of proteins on the activity of the succinic systems in different preparations

The activation by denatured globin of a heart-muscle preparation oxidizing succinate was recorded in a previous paper (Keilin & Hartree, 1947b) under a single set of experimental conditions. Further work revealed that other proteins exhibited the same property, and that the magnitude of the effect was greatly dependent upon phosphate concentration. Such experiments showed that, except at very high [phosph], the activity of a heart preparation in presence of added proteins was independent of [phosph] and that the effect of protein in sub-optimal [phosph] was to raise the activity of the preparation to the optimal level (Table 3). From

Table 3. *The effect of phosphate concentration on the oxidation of succinate by a heart preparation $\pm$ added protein*

(Oxidase (0.04 ml.); succinate and cytochrome c; 0.3 ml. 1.4% denatured globin or 0.3 ml. horse plasma; temp., 39°; pH, 7.3.)

Phosphate conc. (M)	O ₂ uptake (μ l./30 min.)		
	Alone	+ Plasma	+ Globin
0.03	198	320	349
0.09	294	348	—
0.15	325	340	331
0.30	279	279	—

this it follows that the effect of proteins can only be studied at low [phosph] and the concentration chosen for subsequent work was 0.07 M. It is important to note that denatured globin is completely precipitated at the pH of our experiments (7.3). From the results of experiments carried out

with an ox-heart preparation and summarized in Table 4 it is evident that proteins generally, but not their digests, activate the oxidation of succinate, and that denatured and consequently insoluble proteins are the most efficient in this respect. Similar results were obtained with a horse-heart preparation.

Table 4. *The effects of various substances on the aerobic oxidation of succinate by a pig-heart preparation*

(Phosphate (0.07M); oxidase (0.05 ml.); succinate, cytochrome *c*; temp., 39°; pH, 7.3.)

Substance added	Relative rate of O ₂ uptake
None	100
Crystalline serum albumin (2%)	219
Peptone (2%)	117
Gelatine (2%)	200
Casein (commercial (2%))	171
Tryptic digest of casein (2%)	122
Horse plasma (0.4 ml.)	210
Methaemoglobin (2%)	206
Denatured globin (2%)	285*
Boiled methaemoglobin (2%)	244*
Ca ₃ (PO ₄) ₂ gel (30 mg.)	199
CaCl ₂ + AlCl ₃ (each 4 × 10 ⁻⁴ M)†	165
Boiled oxidase (0.1 ml.)	167
Vacuum-dried oxidase (4 mg.)	180‡

* Precipitate of protein in manometer flask.

† Concentrations used by Schneider & Potter (1943).

‡ Corrected for O₂ uptake of dried preparation.

Pigeon-breast-muscle preparations, on the other hand, are not affected by proteins (Keilin & Hartree, 1947b).

D. *The effect of cytochrome c compared with that of indifferent proteins*

The increased rate of oxidation of succinate by heart-muscle preparations obtained on adding proteins is of the same order as that given by cytochrome *c*. This raises the question whether cytochrome *c* may act as an indifferent protein as well as a specific oxido-reduction catalyst. The study of this question is most conveniently carried out with certain modified types of preparations which will be described below.

(1) *Experiments with frozen heart-muscle preparations.* The best results were given by a pig-heart preparation preserved 2-3 days at 5° after freezing 3 times in an ice-salt mixture. The rate of oxidation of succinate by this preparation was studied in presence of (a) horse-blood serum, (b) cytochrome *c* (0.43% Fe), (c) alkali denatured cytochrome *c*, (d) denatured globin and (e) denatured globin treated with alkali in the same way as the cytochrome *c*. The alkali treatment consists in 18 hr. contact with N-NaOH followed by neutralization with HCl and 4 hr. dialysis to remove excess of salt. We have previously shown (Keilin & Hartree, 1940) that such treatment renders cytochrome *c* catalytically inactive in an oxidizing system obtained from

tissue extracts. The results of these experiments are summarized in Fig. 2 where the activating effects of various proteins are plotted against log protein concentration. The order of efficiency is: cytochrome *c* > denatured globin > serum proteins, but whereas alkali treatment reduces cytochrome *c* to the serum level, it does not affect denatured globin. These experiments distinguish clearly between the influence of added cytochrome *c* as an indifferent protein and its catalytic effect as an essential oxido-reduction catalyst. No explanation of the maxima in certain curves is forthcoming from our experiments.

Fig. 2. Effect of cytochrome *c*, denatured globin and serum proteins on the oxidation of succinate by a frozen heart-muscle preparation (1.1 mg.). Ordinates: activity expressed as percentage of that in absence of the various proteins (36 μ l. O₂/10 min.). Abscissae: log percentage protein concentration. Experiment carried out at 39° in 0.07M-phosphate (pH 7.3).

(2) *Experiments with vacuum-dried heart-muscle preparations.* Horse-heart preparation (20 ml.) yielded 832 mg. powder on drying in the manner previously described. It was preserved in a desiccator for periods up to 4 weeks and resuspended in water (41.6 mg./ml.) as required. For manometric experiments 0.05 ml. of this suspension was used, while control runs were carried out with the corresponding amounts of undried material. The effect of phosphate concentration on this preparation is shown in Fig. 1. The results collected in Table 5 show that the activity of the preparation towards succinate is very much reduced by drying, when tested in 0.07M-phosphate, and it is not re-activated by cytochrome *c*. Addition of denatured globin, however, increases the activity of the dried material to that obtained at the optimum phosphate concentration, and the addition of cytochrome *c* at this stage leads to a further increase in O₂ uptake. Cytochrome *c*, therefore, does not act here as an indifferent protein. An analogous oxidase

preparation from ox heart was less markedly affected by drying, probably owing to the presence of proteins which may function as denatured globin. In both cases, however, the effects produced by cytochrome *c* and by globin are additive. The results obtained with dried oxidase preparations in experiments of the type set out in Table 5 can vary considerably from one preparation to another and with the age of the preparation. Nevertheless, the distinction between the general effect of proteins and the specific effect of cytochrome *c* always remains clear.

Table 5. *Oxidation of succinate by heart-muscle preparations before and after drying. Effect of cytochrome c and denatured globin*

(Globin (0.3 ml. 1.4 %); 0.07 M-phosphate buffer; temp., 39°; pH, 7.3.)

	O ₂ uptake (μl./15 min.)		
	Horse heart		Ox heart (dried)
	Fresh	Dried	
Oxid. + succ.	78	2	62
Oxid. + succ. + cyt. <i>c</i>	98	9	102
Oxid. + succ. + denat. globin	193	80	145
Oxid. + succ. + denat. globin + cyt. <i>c</i>	220	174	252

(3) *Experiments with horse-kidney preparations.* The characteristic property of this type of preparation is that it oxidizes succinate or *p*-phenylenediamine very slowly unless cytochrome *c* is added, when the rate of O₂ uptake may increase at least 5-fold (Table 2). We have previously noted this marked effect of added cytochrome *c* upon kidney preparations and have suggested that it is due to a deficiency of cytochrome *c* (Keilin & Hartree, 1940). When the cytochromes in our kidney preparations are examined spectroscopically after reduction with succinate or Na₂S₂O₄, a feeble band (*a*) is seen together with a broad diffuse band (*b*₁), which occupies the same position as the two distinct bands (*b*) and (*c*) found in heart preparations. In the same paper we showed that it was possible to obtain heart preparations that were not deficient in cytochrome *c* and yet were virtually inactive in the aerobic oxidation of succinate unless soluble *c* was added. It is thus possible for *c* to be present in an unavailable form. In this connexion the results of Slater (1949) are of interest. He has found that if the acidification to pH 5.5 is avoided during the preparation of the kidney oxidase (see p. 206) and replaced by high-speed centrifuging as a means of concentrating the active material, a normal heart-type cytochrome spectrum can be observed on addition of succinate. However, the rate of oxidation of succinate by such material, which shows an optimal effect of [phosph], is also low in absence of added *c*; hence the *c* in the preparation must be largely inaccessible to other components of the catalytic system.

Experiments with our kidney preparation, under standard experimental conditions, are shown in Table 6. The distinction between the accelerating effects of cytochrome *c* and denatured globin is apparent from the fact that globin is ineffective in absence of added *c*.

Table 6. *The effect of various activating factors on the oxidation of succinate by a horse kidney preparation*

(Denatured globin (0.3 ml. 1.4 %), 4×10^{-4} M-AlCl₃, 10^{-3} M-methylene blue; 0.12 ml. kidney preparation, 0.07 M-phosphate buffer; temp., 39°; pH, 7.3.)

Factor added	O ₂ uptake (μl./30 min.)
None	15
Denatured globin	22
AlCl ₃	21
Meth. blue	54
Cyt. <i>c</i>	111
Cyt. <i>c</i> + globin	172
Cyt. <i>c</i> + AlCl ₃	155

E. *Effect of metals on the oxidation of succinate by different preparations*

Horecker, Stotz & Hogness (1939) have shown that low concentrations of salts of Al⁺⁺⁺, Cr⁺⁺⁺ and the rare earths markedly increase the rate of oxidation of succinate in presence of an ox-heart preparation. Swingle, Axelrod & Elvehjem (1942) found a similar activation of various tissue preparations by Ca⁺⁺ (see p. 210) while Schneider & Potter (1943) have studied the activation of an unwashed liver preparation by Ca⁺⁺ and Al⁺⁺⁺.

(1) *Effect of Ca⁺⁺ and Al⁺⁺⁺.* The influences of these metallic ions added separately on the aerobic oxidation of succinate by heart-muscle preparations are quite different from those recorded by Schneider & Potter who found that, in liver extracts, Ca⁺⁺ alone activated the system and that Al⁺⁺⁺ merely abolished an initial lag in O₂ uptake. In Fig. 3

Fig. 3. Effect of different concentrations of CaCl₂ and AlCl₃ on the rate of O₂ uptake by 0.1 ml. heart-muscle preparation in presence of cytochrome *c* and succinate. 0.07 M-phosphate, pH 7.3; temp., 39°. Abscissae: log [CaCl₂] or [AlCl₃] expressed as molar concentrations.

are shown the effects of various concentrations of CaCl₂ and AlCl₃ on the aerobic activity of a horse-heart preparation under the standard conditions. It was of interest to find that the concentration at which the effect of Ca⁺⁺ disappears

is also the minimum concentration at which a gelatinous precipitate of $\text{Ca}_3(\text{PO}_4)_2$ is formed in the manometer flask. In the case of Al^{+++} the minimum concentration could not be gauged as the precipitate forms very slowly at low Al^{+++} concentration. At $4 \times 10^{-4}\text{M}$, only Al^{+++} is effective and the activation by the ions was found not to be additive. Anaerobic experiments with methylene blue gave no useful results owing to co-precipitation of the dye with the insoluble phosphate. The fact that the effects produced by $\text{Ca}^{++} + \text{Al}^{+++}$ and by globin are not additive suggests that their modes of action are similar. They both form precipitates in the manometric flasks, thus providing surfaces for possible adsorption, which corroborates the conclusion reached by Horecker *et al.* (1939), namely that the activation they obtained with Cr^{+++} and other ions arose from the formation of precipitates of heavy metal phosphates. The dried oxidase preparations are also markedly reactivated by Ca^{++} and Al^{+++} . Different results, however, were obtained when $\text{CaCl}_2 + \text{AlCl}_3$ were added to the oxidase preparation before drying. In such experiments, to 10 ml. heart-muscle preparation were added 0.43 ml. each of 0.6M- CaCl_2 and 0.6M- AlCl_3 in order that the final concentration of the two salts should be $4 \times 10^{-4}\text{M}$ in the subsequent manometer experiments. After vacuum drying and resuspension of the powder in 10 ml. water, the preparation was found to be completely inactive and further addition of Ca^{++} and Al^{+++} failed to reactivate it, whereas the control dried preparation behaved normally (Table 7).

Table 7. *Activity of succinic system in horse-heart preparations dried with or without the addition of CaCl_2 and AlCl_3*

	$(4 \times 10^{-4}\text{M-}\text{CaCl}_2 \text{ and } \text{AlCl}_3; \text{ temp., } 39^\circ; \text{ pH, } 7.3.)$
	$\text{O}_2 \text{ uptake}$ ($\mu\text{l./20 min.}$)
Oxid. dried with Ca^{++} and Al^{+++} + succ. + cyt. c	0
Oxid. dried with Ca^{++} and Al^{+++} + succ. + cyt. c + $\text{CaCl}_2 + \text{AlCl}_3$	0
Oxid. dried alone + succ. + cyt. c	17
Oxid. dried alone + succ. + cyt. c + $\text{CaCl}_2 + \text{AlCl}_3$	109

Table 8. *Effect of metals on the succinic system in horse heart and pigeon breast-muscle preparations*

(Heart preparation (0.05 ml.), 0.07 ml. pigeon preparation, 0.2 ml. cytochrome c in all cases. Metals ($2 \times 10^{-3}\text{M}$) added as chlorides or sulphates; 0.07M-buffer; temp., 39° ; pH, 7.3.)

Metal added	Preparation and buffer					
	Heart prep. in phosphate		Heart prep. in glyoxaline		Pigeon prep. in phosphate	
	Rate*	Ppt.†	Rate*	Ppt.†	Rate*	Ppt.†
None	(100)	—	(100)	—	(100)	—
Ba^{++}	171	<i>I</i>	96	‡	88	<i>I</i>
Ca^{++}	208	<i>I</i>	108	<i>O</i>	86	<i>I</i>
Ce^{+++}	181	<i>I</i>	0	<i>D</i>	85	<i>I</i>
Co^{++}	68	<i>S</i>	0	<i>D</i>	43	<i>S</i>
Cu^{++}	0	<i>I</i>	0	<i>D</i>	0	<i>I</i>
Fe^{+++}	174	<i>I</i>	86	§	94	<i>I</i>
Li^+	104	<i>O</i>	109	<i>O</i>	100	<i>O</i>
Mn^{++}	175	<i>I</i>	109	<i>O</i>	110	<i>I</i>

* Relative initial rates of O_2 uptake.

† Key to letters and signs: *O*, no precipitate formed during experiment; *I*, immediate precipitate of gelatinous phosphate; *S*, slow formation of precipitate; *D*, slow formation of dense granular precipitate; ‡, slight crystalline precipitate of BaSO_4 ; §, precipitate of $\text{Fe}(\text{OH})_3$.

Our results cannot be interpreted in the same manner as those of Swingle, Axelrod & Elvehjem (1942). By working with homogenates of unwashed tissues, these workers obtained evidence that the effect of Ca^{++} arises from its activation of an enzyme hydrolyzing coenzyme I, the latter being an indirect inhibitor of succinic dehydrogenase (see p. 215). In fact we were able to show that a filtrate of boiled heart preparation was completely devoid of this coenzyme since it could not replace authentic coenzyme I in the re-constructed muscle lactic dehydrogenase system (Straub, 1940). Further arguments against such a possibility are: (1) that heart-muscle preparations in optimal [phosph] are not affected by Ca^{++} since the latter merely raises the activity to the optimal level, and (2) no effect of Ca^{++} can be observed when using enzyme material from other sources or when experiments are carried out in phosphate-free buffers (Table 8).

(2) *Effects of other metals.* The influence of other metals on the activity of a heart preparation in phosphate and in glyoxaline buffers and on a pigeon preparation in phosphate buffer is summarized in Table 8. In no case was there any activation of heart preparations in glyoxaline buffer or of pigeon preparations in phosphate buffer. The activations produced by Ba^{++} , Fe^{+++} , Mn^{++} and Ce^{+++} resemble those obtained with Ca^{++} and Al^{+++} . Inhibition is always complete in the case of Cu^{++} , but variable in the case of Co^{++} . The formation of dense granular precipitates by Ce^{+++} , Co^{++} and Cu^{++} in glyoxaline accompanies complete inactivation. The lack of activation by metals of the pigeon preparation in phosphate buffer finds a parallel in the absence of any effect of proteins, whether native or denatured, under the same circumstances.

F. Evidence for the existence of a carrier linking succinic dehydrogenase with cytochrome

Several workers claim to have demonstrated the existence of, or even to have isolated, a carrier linking succinic dehydrogenase with the cytochrome system. We shall now summarize the evidence

brought forward in support of these claims and re-examine it in the light of our experiments.

(1) The first enzyme preparation with properties suggesting the existence of a carrier was obtained by Hopkins, Lutwak-Mann & Morgan (1939). They washed a mince of pig heart with sodium chloride and lithium chloride solutions and extracted it with bile salts. After precipitation of this extract with ethanol and extracting the precipitate with urea, a suspension was obtained which did not reduce cytochrome *c*, and which did not catalyze the oxidation of succinic acid by O_2 unless methylene blue was added. Unfortunately, this treatment denatures the components *a*, *a₃* and *b* of cytochrome and inactivates the major part of the succinic dehydrogenase. In fact, the Q_{O_2} of this preparation was about 12 whereas the Q_{O_2} of an active heart preparation lies between 500 and 700. It has been shown (Keilin & Hartree, 1940) that 10 min. contact with 50% ethanol brings about the following changes in our heart muscle preparation: (1) the cytochrome oxidase activity, as measured by its ability to oxidize *p*-phenylenediamine, is abolished, (2) cytochromes *a*, *a₃* and *b* are found, on spectroscopic examination, to be irreversibly modified, and (3) the succinic dehydrogenase activity, measured by the rate of O_2 uptake in presence of methylene blue, is reduced to about one third of its original value. The behaviour of the preparation of Hopkins *et al.* (1939) is thus best explained as being due to inactivation of the cytochrome system rather than to removal of an essential factor.

(2) By ultracentrifuging a heart-muscle preparation, Stern & Melnick (1939) obtained a preparation incapable of oxidizing succinic acid in presence of cytochrome *c*, although it contained an active succinic dehydrogenase, oxidizing the substrate by means of methylene blue, and an active cytochrome system as measured by the rate of oxidation of hydroquinone. However, when the supernatant fluid was added to the ultracentrifuged preparation, a complete system was obtained which catalyzed the aerobic oxidation of succinic acid. The supernatant fluid, according to them, contained 'a supplementary principle' or 'activator' which is a protein of molecular weight about 140,000. As we have mentioned previously (Keilin & Hartree, 1940), we were not in a position to repeat this experiment, but on centrifuging our heart preparation at a lower speed (13,000 r.p.m.) we obtained a sediment which was as active as the original material. As any factor which remained in solution at the higher speed must also have remained at our lower speed it is impossible to reconcile the two experiments on the basis of the separation of a component. The inactivation of Stern & Melnick's ultracentrifuged preparation cannot, therefore, be considered as being due to the separation of a

carrier. It is probable that, under the influence of a high gravitational field, the preparation of Stern & Melnick underwent changes similar to those caused by drying or freezing. No such effect was observed in the lower gravitational field of our experiment.

We have already seen that marked activation of various types of enzyme preparation may be obtained with indifferent and denatured proteins. That Stern & Melnick's results may find an explanation along the same lines appears probable as a result of our experiments with dried heart and kidney preparations. We have shown (Table 5, second column of figures) that on drying a horse-heart preparation its catalytic activity towards succinic acid in presence of added cytochrome *c* is reduced to about 9% of that of the original material. In contrast to the inappreciable effect of cytochrome *c* on the dried preparation, the addition of denatured globin markedly raises the activity, and the further addition of cytochrome *c* at this stage more than doubles the activity so that it approaches 80% of that of the fresh preparation. The analogy with the results of Stern & Melnick becomes more striking when the activities of dried heart and kidney preparations are examined in presence of methylene blue, cytochrome and denatured globin. The results obtained with these heart and kidney preparations are shown in Table 9 and Fig. 4 respectively. It is seen that the activation by cytochrome *c* in such cases is very small and considerably less than that by methylene blue. In other words, a dried preparation catalyzes the oxidation of succinate by methylene blue (Fig. 4, iv) more efficiently than by cytochrome *c* (iii). In absence of added cytochrome *c* globin has no effect on this system (i and ii), but the simultaneous addition of globin and cytochrome *c* leads to a very marked activation (v). Whereas the addition of methylene blue to this last system does not increase its catalytic activity, an increase is obtained when either cytochrome *c*, globin, or both are absent (ia, iia, iiia). Thus a non-specific and denatured protein appears to facilitate the reaction between dehydrogenase and the cytochrome system in the same way as the supplementary principle or activator of Stern & Melnick. The latter cannot, therefore, be considered, without further evidence, as a specific component of the catalytic system in tissues which oxidize succinic acid.

(3) Straub (1942) treated an extract of pig-heart muscle with sodium cholate and ammonium sulphate and resuspended the resulting precipitate in phosphate buffer. This 'cholate preparation' oxidized *p*-phenylenediamine, but not succinate unless methylene blue was added. It thus contained both the cytochrome system and succinic dehydrogenase, but apparently a certain factor linking the two was missing. A material which fulfilled this function

('SC factor') was obtained by heating a portion of the heart extract in alkaline solution. Straub considers the 'SC factor' to be an essential intermediate link in the cytochrome-succinic dehydrogenase system. This factor must be very different from the thermolabile protein 'activator' described by Stern & Melnick.

Table 9. *The effect of methylene blue and denatured globin on the succinic system in a dried horse-heart preparation*

(Denatured globin (0.3 ml., 1.4%), 10^{-3} M-methylene blue; dried oxidase, succinate, cytochrome c, 0.07M-phosphate; temp., 39°; pH, 7.3.)

Addition	O ₂ uptake (μ l./15 min.)
None	11
Meth. blue	58
Globin	160
Globin + meth. blue	184

Fig. 4. Effect of cytochrome c (0.2 ml., 1%), denatured globin (0.3 ml., 1.4%) and methylene blue (10^{-3} M) on the oxidation of succinate by a dried kidney oxidase preparation. Oxidase preparation equivalent to 0.1 ml. of material before drying. 0.07M-phosphate buffer, pH, 7.3; temp., 39°.

We isolated the cholate preparation of Straub strictly according to his description, and we obtained his 'SC factor' by incubation of the heart-muscle preparation for 30 min. at pH 9 and 20° instead of at the higher temperature given in his paper which, in our hands, yielded a completely inactive material. Having confirmed Straub's results (Table 10, 1-4) we attempted to replace the 'SC factor' by substances which activated our enzyme preparations. Calcium phosphate was found to be much more efficient than the SC factor, whereas

denatured globin or Al^{+++} were completely ineffective (Table 10, 5-8). Such a striking difference between calcium phosphate and other activators was not observed with any of the enzyme preparations previously examined. The marked activating effect of Ca^{++} in this case is apparently due to precipitation as calcium salt of the residual cholate. The low activity in Exp. 1 can, therefore, be reasonably ascribed to an inhibition of the catalytic system by sodium cholate present in this preparation. A notable increase in the activity of the 'SC factor' was found after 3 days' storage at 5°, although the dehydrogenase activity of the cholate preparation

Table 10. *The effect of Ca^{++} and denatured globin on the succinic system prepared according to Straub (1942)*

(Cholate preparation (0.5 or 1.0 ml.), 1.0 ml. 'SC factor', 0.2 ml. 1% cytochrome c, 0.04M-sodium succinate, 0.3 ml. $\text{Ca}_3(\text{PO}_4)_2$ gel (20 mg. dry wt.), 0.3 ml. 1.4% denatured globin, 10^{-3} M-methylene blue; temp., 39°; pH, 7.3.)

	O ₂ uptake (μ l./10 min.)	
	Tested same day using cholate prep. (1.0 ml.)	Tested 3 days later using cholate prep. (0.5 ml.)
1. Chol. prep. + cyt. c + succ.	7*	2
2. Chol. prep. + cyt. c + succ. + 'SC factor'	31†	92
3. 'SC factor' + cyt. c + succ.	0	2
4. Chol. prep. + meth. blue + succ.	342	145
5. As 1 + $\text{Ca}_3(\text{PO}_4)_2$	232†	—
6. As 1 + denatured globin	2	—
7. As 2 + $\text{Ca}_3(\text{PO}_4)_2$	231	—
8. As 2 + denatured globin	33	—

* This system is not activated by 10^{-3} M- AlCl_3 nor by increasing [phosph].

† O₂ uptake slows down after first 10 min.

decreased during the same interval. This change coincides with an increased aggregation and precipitation of the proteins in the factor which may then act as a more efficient adsorbent of cholate. It is, therefore, probable that the 'SC factor' does not represent a specific link in the catalytic chain of the system oxidizing succinate, but that it merely serves to remove cholate from solution. On the other hand, since we obtained the 'SC factor' under conditions that were slightly different from those specified by Straub, further study will be required before the mechanism of its action can be definitely established (see also Slater, 1949).

(4) By fractionating an aqueous extract of minced ox or rabbit liver with ammonium sulphate Stoppani (1947) isolated a preparation which, although it contained both succinic dehydrogenase and the complete cytochrome system, was yet incapable of catalyzing the aerobic oxidation of

succinate even in presence of added cytochrome *c*. This material was precipitated from the liver extract within the range 0.0–0.2 saturation with ammonium sulphate. However, when the fraction which precipitated between 0.33 and 0.6 saturation was purified through calcium phosphate it was found to have a strong activating effect on the first fraction in the aerobic oxidation of succinate. One of the peculiarities of the first fraction as a succinic dehydrogenase was that it could utilize ferricyanide as H acceptor but not methylene blue. The activator was easily destroyed by heat and by acids and would appear to have been a protein. It could not be replaced by 10^{-5} M- Ca^{++} or Al^{+++} or by different coenzymes.

In our attempts to repeat Stoppani's experiments, following his methods as closely as possible, we were

(arrows indicate direction of electron transfer)

unable to obtain fractions which showed a differentiation in the reactions with ferricyanide and methylene blue: in fact all fractions that we obtained between 0 and 0.2 saturation readily reduced methylene blue anaerobically in presence of succinate. On one occasion we were able to obtain from rabbit liver two fractions precipitated within the specified ammonium sulphate concentrations which resembled Stoppani's materials. Details of this experiment are set out in Table 11, where it is seen that the factor may be replaced by Ca^{++} or horse serum but not by Al^{+++} or denatured globin. We have used much higher concentrations of Ca^{++} and Al^{+++} than did Stoppani, which may well explain the discrepancy (cf. Fig. 3).

Table 11. *Effect of Ca, Al and proteins on the succinic system prepared according to Stoppani (1947)*

(Enzyme preparation, rabbit liver (0.5 ml.), 1 ml. 'factor' (rabbit liver), 0.2 ml. 1% cytochrome *c* (in all experiments), 10^{-3} M-methylene blue, 0.005 M- AlCl_3 , 0.005 M- CaCl_2 , 0.14% denatured globin, 0.3 ml. horse serum, 0.04 M-sodium succinate, 0.07 M-phosphate buffer; temp., 39°; pH, 7.3.)

	O_2 uptake ($\mu\text{l./hr.}$)
1. Enzyme + succinate	3
2. 'Factor' + succinate	3
3. Enzyme + 'factor' + succinate	124
4. As 1 + Ca^{++}	107
5. As 1 + Al^{+++}	8
6. As 1 + serum	87
7. As 1 + denatured globin	21
8. As 1 + meth. blue	76

Our experiments suggest that the activating material isolated by Stoppani contains no essential link in the catalytic system oxidizing succinate, but comes within the category of indifferent proteins

which can have a marked effect upon our heart-muscle and kidney preparations.

(5) Yakushiji & Okunuki (1940) have described a haem-protein of haemochromogen type under the name cytochrome *c*₁ and consider it to be a catalytic intermediate in the cytochrome system. They repeatedly washed an isoelectric precipitate of a heart preparation with acetate buffer, pH 4.6, before suspending it in phosphate at pH 7.5 and found that, on reduction with succinate + HCN, it showed an absorption band at 552 m μ . This pigment, which can be extracted from heart preparations in a soluble form, will reduce cytochromes *a* and *c*. These authors suggest that it acts as an oxidation-reduction carrier between cytochrome *b* and cytochromes *a* and *c*:

However, since *c*₁ can only be detected or isolated after treatments which have an adverse effect upon the activity of the complete succinic system (e.g. sodium cholate or ether) the possibility that it is an artifact derived from other haem-proteins (see p. 214) must be seriously considered. On the other hand, the function postulated for this substance is very similar to that put forward by Slater (see below) for a labile haem-protein detected by him in heart-muscle preparations, and it is possible that the two 'factors' may be closely related.

(6) While studying the effect of BAL (2:3-dimercaptopropanol) on the succinic dehydrogenase-cytochrome system, Slater (1948, 1949) has obtained a heart-muscle preparation exhibiting interesting properties. Although this preparation failed to catalyze the aerobic oxidation of succinic acid, it showed an almost unimpaired activity both of succinic dehydrogenase, as shown by the rate of oxidation of succinate in presence of methylene blue, and of the cytochrome system estimated by the oxidation in presence of cytochrome *c* of ascorbic acid, hydroquinone or *p*-phenylenediamine. On addition of succinate to such a preparation, cytochrome *b* becomes permanently reduced, whereas the other cytochrome components remain in the oxidized state, an effect resembling that observed on addition of urethane to a normal preparation (Keilin & Hartree, 1939, 1940). On addition of $\text{Na}_2\text{S}_2\text{O}_4$, the reduced components of cytochrome (*a*, *a*₃, *b*, *c*) appear normal, although the total protohaematin content of the preparation, determined by addition of pyridine plus $\text{Na}_2\text{S}_2\text{O}_4$ and measurement of the intensity of the haemochromogen band at 548–560 m μ , is 20% below that of the heart preparation before treatment with

BAL. Slater's interpretation of these results is that the heart preparation contains a BAL-labile factor which is an intermediate in the electron transfer from cytochrome *b* to cytochrome *c* and which is probably a haematin derivative. The system may be represented as follows:

The preparation obtained by Slater shows some resemblance to two preparations that we obtained by incubating heart-muscle oxidase either at pH 5.0 or at a higher pH in presence of pancreatin (Keilin & Hartree, 1940). In all cases there appears to be a lack of interaction between the succinic dehydrogenase on the one hand and the cytochrome system on the other. We suggested that the impairment of the aerobic succinoxidase activity could be explained, either by loss of a factor intermediate between dehydrogenase and cytochrome *c* or by an irreversible change in the colloidal structure of the preparation, with a consequent decrease in the mutual accessibility of the catalysts in the system.

Although we cannot exclude the possibility of the existence of several further intermediate links between the known components of the succinic dehydrogenase-cytochrome oxidase system, we have seen that much of the evidence is inconclusive, and so far only that brought forward by Slater appears to be well founded upon experimental evidence.

DISCUSSION

The tissue preparations used for the study of the system catalyzing the aerobic oxidation of succinic to fumaric acid consist of colloidal suspensions of protein aggregates. In addition to the complete succinic dehydrogenase-cytochrome system these preparations also contain fumarase together with small concentrations of catalase and certain dehydrogenases (e.g. malic and lactic); they are, however, free from haemoglobin and coenzymes I and II. Being completely devoid of substrate, they have no O_2 uptake, show cytochromes in a completely oxidized state and do not reduce methylene blue anaerobically. After addition of sodium succinate the cytochromes rapidly become reduced and on aerating the mixture they re-oxidize only to become again reduced when aeration ceases. The preparation will now take up O_2 rapidly and will reduce methylene blue anaerobically.

Spectroscopically the preparation shows the cytochrome components *a*, a_3 , *b* and *c* (Keilin & Hartree, 1939). It also contains other forms of haematin which were previously described as free haematin (Keilin, 1925), but which we now consider to be present as one or more haematin-protein derivatives (Keilin & Hartree, 1947*b*). That such compounds are not visible spectroscopically may be due to their

diffuse absorption bands. However, on treating these preparations with $\text{Na}_2\text{S}_2\text{O}_4$ and pyridine, the extra haematin becomes spectroscopically visible as pyridine haemochromogen, the α band of which is clearly seen obliterating the space between the α bands of cytochromes *b* and *c*.

Since the activity of the complete succinic system can be studied both spectroscopically and manometrically, we are able to localize the actions of a number of inhibitory or activating factors and to determine the mechanism of their action. Further analysis of this system can be made by independent study of the activities of its two portions. Thus the activity of succinic dehydrogenase can be determined either anaerobically by the rate of reduction of methylene blue, or aerobically, by the rate of O_2 uptake in presence of methylene blue and of cyanide which inhibits the cytochrome oxidase. The activity of the cytochrome system can readily be followed manometrically by the rate of oxidation of substances which reduce cytochrome *c* directly, i.e. without the need of specific enzymes. Such substances are ascorbic acid, cysteine, phenols and aromatic amines.

Whereas some factors act on one or other of the known components of the system others affect either some, as yet undetermined, intermediate components, or the colloidal structure of the preparation which assures the mutual accessibility of the components. In any attempt to analyze the complex systems represented by our colloidal preparations it is important to determine not only the point at which the factor reacts but also its mode of action. Even if we confine our attention to inhibitors we find that in some cases the mode of action is easily demonstrated, while in others it requires a more thorough investigation. Thus the powerful, instantaneous and perfectly reversible inhibition of the system by HCN, NaN_3 or H_2S is due to their combination with the trivalent Fe of cytochrome oxidase (a_3), while the effect of CO depends upon its ability to combine with oxidase Fe in the divalent state. All these reactions can be followed spectroscopically (Keilin & Hartree, 1939). On the other hand, malonate (Quastel & Wooldridge, 1928), oxaloacetate (Das, 1937) and pyrophosphate (Dixon & Elliott, 1929; Leloir & Dixon, 1937) act as competitive inhibitors of succinic dehydrogenase. The relative affinity of this enzyme (*E*) for substrate (*s*) and inhibitor (*i*) determined experimentally and expressed as a partition constant $K = [Ei][s]/[Es][i]$ is about 13 for malonate, 23 for pyrophosphate and 850 for oxaloacetate. Spectroscopic study of enzyme preparations shows that these inhibitors prevent the reduction of all components of cytochrome by succinate.

On the other hand the mechanisms of the inhibition of this system by coenzyme I and by sodium diethyldithiocarbamate (DDC) are less direct and require a more careful consideration. The inhibition of aerobic oxidation of succinate by coenzyme I was first recorded by Potter (1939). We confirmed this effect and found a marked inhibition at coenzyme concentrations as low as 0.5×10^{-5} – 2.5×10^{-5} M. At first sight this inhibition could be ascribed to a direct reaction of coenzyme with some reactive groups of the dehydrogenase. However, a study of this reaction under varying conditions revealed the following mechanism (Keilin & Hartree, 1940). Since heart-muscle preparations contain fumarase and malic dehydrogenase, part of the fumarate arising from the oxidation of succinate is transformed to malate which, in presence of coenzyme, is oxidized to oxaloacetate. The latter, being neither reduced nor decarboxylated at an appreciable rate, acts as a competitive inhibitor of succinic dehydrogenase. In fact, if the concentration of oxaloacetate reaches only 0.2 % of that of the succinate it would already inhibit the O_2 uptake by > 50 % (see p. 214).

The study of the inhibition by DDC is of special interest. This substance is chiefly known as a reagent for the colorimetric estimation of copper and will act as an inhibitor of reactions catalyzed both by Cu^{++} ions and by copper-protein enzymes such as polyphenol oxidase. The inhibition by this substance of the aerobic oxidation of succinate at first suggested the possibility that a copper derivative participated in the reaction. However, a further study of this reaction clearly showed that the inhibition was not due to DDC itself but to its oxidation product tetraethyldithiocarbamyl disulphide (TDD) $(C_2H_5)_2NCS.SS.SCN(C_2H_5)_2$ derived from DDC through an oxidation catalyzed by the cytochrome system, since DDC reduces cytochrome *c* (Keilin & Hartree, 1940). In fact it was found that while DDC inhibited succinate oxidation only after it had become oxidized, TDD, in concentrations as low as 5×10^{-6} and 2×10^{-5} M, inhibited O_2 uptake immediately to the extent of 70 and 100 % respectively.

Since the demonstration by Hopkins & Morgan (1938) that the activity of succinic dehydrogenase depends upon the integrity of certain —SH groups in the enzyme, the mechanism of action of a number of inhibitors has been explained by their ability to combine with —SH groups or to oxidize them (see Potter & DuBois, 1943; Slater, 1949).

The activity and behaviour of the succinic system show considerable variations both with the type of tissue used and with the species of animal from which it was obtained. The effects of different factors, such as phosphate concentration, native and denatured proteins and salts of a number of metals,

were tested both on fresh enzyme preparations and on vacuum-dried and frozen samples.

The study of the influence of phosphate concentration on the activity of a heart preparation has demonstrated a relationship between activity and the degree of colloidal dispersion. We have not attempted to express the degree of dispersion in any quantitative form, but have regarded the time taken for the proteins in a preparation to flocculate spontaneously and settle out under gravity as a rough measure of the degree of dispersion. Thus the diluted heart-muscle extract described on p. 206, which remains homogeneous for several hours, is regarded as completely dispersed. Maximum dispersion and enzyme activity are obtained in 0.15 M-phosphate (pH 7.3) with an increasing flocculation and falling off in activity as the concentration deviates from this figure. When the total salt concentration remains constant and [phosph] is varied, an activating effect of phosphate is detectable and the occurrence of an optimum concentration is apparently due to two opposing influences: peptization and salting out. In our kidney preparation the former influence is absent; hence the activity increases continuously with diminishing [phosph]. A point of certain interest is that fully active heart preparations can be obtained in absence of phosphate, glyoxaline replacing phosphate during the extraction from the muscle and in the manometers.

We have shown that, contrary to the views of Biró & Szent-Györgyi jun. (1946), ATP does not activate, but slightly inhibits the system oxidizing succinic acid. If any link exists between this system and ATP it must be indirect and may depend upon catalytic systems not present in our preparations. Moreover, even if the synthesis of ATP is linked with the oxidation of succinate it does not logically follow that ATP should have any effect upon the activity of the succinic dehydrogenase-cytochrome system.

The importance of colloidal structure for the activity of the complete system is clearly demonstrated by the fact that when [phosph] is reduced to a suboptimal value, e.g. 0.07 M, the addition of denatured globin or of Ca^{++} or Al^{+++} brings the activity of the system to the level shown at the optimum [phosph] (Table 3). These additions give rise to bulky precipitates on which the enzyme preparation becomes adsorbed although similar, but less marked, effects are observed with proteins that remain in solution (Fig. 2 and Table 4). The effect of denatured globin is even more striking in the cases of dried heart and dried kidney preparations (Table 5 and Fig. 4). Where the metals do not form bulky precipitates (e.g. in glyoxaline buffer) no activation is obtained while a lack of activation in phosphate buffer by either proteins or metals is observed if the enzyme preparation already consists

of a bulky flocculent precipitate as in the case of pigeon-breast muscle (see Keilin & Hartree, 1947*b*, table 2; also Table 8 of this paper).

We have seen that the activation of the system by proteins generally and by cytochrome *c* may be of the same order of magnitude. This naturally raises the question of the exact function of added *c* in our experiments. That it acts as an oxido-reduction catalyst and not as an indifferent protein is clearly demonstrated by the following observations:

(1) When cytochrome *c* is denatured by treatment with alkali it becomes autooxidizable and, at the same time, catalytically inactive (Keilin & Hartree, 1940). Such material fails to accelerate the O_2 uptake of a heart preparation oxidizing succinate at a concentration equal to the optimal concentration of native *c* (Fig. 2).

(2) Unlike indifferent proteins or metallic ions, cytochrome *c* markedly increases the rate of oxidation of succinate by fresh kidney preparations (Table 6).

(3) The above proteins and ions may activate some preparations to an equal or even greater degree than does cytochrome *c*. However, the effects upon O_2 uptake induced by the indifferent activators and by *c* are additive or even synergic (Table 5).

(4) Dried enzyme preparations from horse heart or kidney show a very low catalytic activity towards succinic acid. The reactivation of a dried horse-heart preparation by denatured globin is usually greater than that produced by cytochrome *c*. However, when both substances are added simultaneously, the resulting activity is markedly greater than the sum of the activities in presence of each of these substances acting separately. The behaviour of such preparations can, however, vary quite widely (see p. 209). A dried kidney preparation (Fig. 4), which fails to oxidize succinate, cannot be reactivated by proteins. Some reactivation is obtained with *c*, but methylene blue is more effective. On the other hand, denatured globin and cytochrome *c* added together will produce a very striking increase in the activity of the dried preparation, and bring it almost to the level of activity of the fresh material with the same additions.

It was previously shown (Keilin & Hartree, 1940, 1945) that the catalytic activity of cytochrome *c* within intact cells and tissues or their colloidal extracts is much greater than that of *c* added in soluble form. The experiments described in this paper lend considerable support to our view that the high activity of the succinic system in such extracts is due largely to the retention within the colloidal particles of the favourable spatial organization of catalysts characteristic of the cell. On such a hypothesis the much lower catalytic activity of added *c* is understandable. Moreover, the loss in activity of a tissue preparation treated in different

ways, even when it can be made good by the addition of *c*, is less likely to be due to loss of endogenous *c* than to changes in colloidal structure which diminish the accessibility of endogenous *c* to other components of the system. The changes resulting from the various treatments can be reversed, and the system as a whole reactivated by addition of factors having an influence on the colloidal structure, such as proteins, ions yielding insoluble phosphates, and changes in [phosph]. An explanation of this reactivation can only be proposed in very general terms. Thus the factors may be responsible either for reorientation of the components, especially of endogenous cytochrome *c*, within the colloidal particles or for providing new reactive surfaces to which catalysts, in particular exogenous *c*, may become anchored. On these grounds the wide variations in response to these factors from one preparation to another are understandable.

The more or less complete loss of accessibility of cytochrome *c* may show different degrees of reversibility. In the extreme case of the dried kidney preparation the removal of *c* from the sphere of influence of other components is almost irreversible since it is impossible to reactivate the succinic system by addition of Ca^{++} or proteins, and, furthermore, added *c* cannot be utilized catalytically unless such factors are also added. This preparation is of special interest since it resembles those obtained by several workers in their attempts to demonstrate the existence of new intermediary links between succinic dehydrogenase and cytochrome *c*. Such preparations share the property of being more strongly activated by methylene blue than by cytochrome *c*, and of being very highly activated when the latter is supplemented by another factor isolated from the same tissue. Although we have seen that it is not necessary to postulate the absence from the dried kidney preparation of a specific link in the succinate oxidizing system, one cannot rule out the possibility that one or more additional links between known components of the succinic dehydrogenase-cytochrome system do, in fact, exist. However, we have shown that the evidence brought forward is inadequate in all cases with one possible exception. The only reasonable evidence is that of Slater (1948), who postulates a specific link, probably of haematin nature, acting as an oxido-reduction carrier between cytochromes *b* and *c*. So far this conclusion is based solely upon an irreversible destruction of the factor and not upon its separation from an active preparation.

The activating effects that we have obtained may be summarized as follows. Marked changes in catalytic activity are associated with the introduction of bulky precipitates. If such precipitates are formed by addition of metallic salts giving gelatinous phosphates, or of proteins, an increase in activity may

be observed. If, on the other hand, precipitates are formed from the enzyme material itself there is generally a loss of activity (e.g. freezing or drying). Lost activity can frequently be restored by formation of precipitates of the activating type.

Treatments which reduce the activity by modifying the colloidal structure may (1) make endogenous cytochrome *c* unavailable or (2) make both endogenous and added *c* unavailable to the rest of the system. It is because the latter effects may often be reversed by indifferent proteins that claims for the existence of new 'links' in the succinic system have been made. Similarly, activation by Ca^{++} of our preparation is not due to an accelerated removal of coenzyme I.

Previous evidence for new 'links' and for the function of Ca^{++} was in each case based upon one type of preparation which was studied under a fixed set of conditions. Our results, however, show that a slight modification of these conditions may, through changes in colloidal structure, lead to fundamentally different results.

We may fittingly conclude by quoting from an earlier paper on the same subject (Keilin & Hartree, 1940). 'The succinic dehydrogenase-cytochrome system even in the cell-free colloidal preparations behaves as a true respiratory system of the cell, showing a high catalytic activity and being affected by all inhibitors in the same way and to the same degree as the normal respiration of intact cells. The efficiency of the system depends, however, not only on the integrity of its components, but also on that of the colloidal structure which supports them and assures their mutual accessibility.'

SUMMARY

1. A study of the succinic dehydrogenase-cytochrome system in various types of tissue extract has been made, with special reference to the effect of colloidal structure on enzyme activity.

2. Heart-muscle preparations show optimum activity in 0.15 M-phosphate; at lower phosphate concentrations addition of proteins or certain metallic ions raises the activity to the optimum level. In this respect insoluble denatured proteins are the most effective. The activity of these preparations falls considerably after freezing, but it can be restored by addition of cytochrome *c*. The pronounced loss of activity that accompanies vacuum drying can be fully reversed only by adding both cytochrome *c* and either proteins or certain metal salts.

3. Kidney preparations are feebly active unless cytochrome *c* is added. They are virtually inactive

after vacuum drying, but can be restored to full activity by addition of both cytochrome *c* and protein, although each added separately has very little effect. The activity of these preparations falls as the buffer concentration is increased; there is no optimum concentration.

4. The activation by metals is obtained in phosphate buffer, where gelatinous precipitates are formed, but not in glyoxaline buffer.

5. It is considered that the catalysts in the colloidal particles, as in the intact cells, are more or less rigidly held together within a framework which ensures their mutual accessibility and a consequent high catalytic activity. Any change in colloidal structure will therefore modify the overall activity without necessarily removing or destroying any individual catalyst.

6. The inhibitory effects of freezing, drying, extreme [phosph], are intimately bound up with the formation from the enzyme material of flocculent precipitates and a consequent decrease in inter-availability. Activation, however, arises when flocculent precipitates of foreign materials (proteins, gelatinous phosphates) are formed in the medium. Such material provides new surfaces upon which reorientation can take place. Since the pigeon preparations already consist of flocculent material they do not undergo activation.

7. It has been clearly demonstrated that added cytochrome *c* acts in these experiments solely as an oxido-reduction catalyst and not as a protein affecting the colloidal structure.

8. The various claims to have demonstrated the existence of new 'links' in the succinic system (comprising dehydrogenase, cytochromes *b*, *c*, *a* and a_3) have been examined in detail. All are shown to be based upon insufficient evidence except that of Slater (1948), who has produced reasoned arguments in favour of an oxido-reduction catalyst linking *b* and *c*.

9. Since our preparations are devoid of coenzyme I the view that its activation by Ca^{++} arises from an accelerated decomposition of this coenzyme, as is the case with fresh homogenates, is untenable. Furthermore, Ca^{++} is ineffective with certain types of tissue preparation.

10. Two types of factor (activating or inhibiting) can be distinguished. They may function either by reacting specifically with a catalyst in the system or by affecting the colloidal structure of the enzyme material. Activation of the latter type has often been misinterpreted as evidence for additional carriers or 'links' in the succinic dehydrogenase-cytochrome system.

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The Component Acids of some Seal Blubber and Liver Fats

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(Received 20 July 1948)

The fatty oils of seals have received little detailed investigation, but their general analytical characteristics as recorded in the literature (cf. Halden & Grün, 1929) reveal considerable variations in the blubber oils, iodine values from 122 to 163 having been reported. This unusually wide range is reflected in the two specimens of seal blubber oil which have so far been examined in greater detail, namely, a commercial sample (probably of Newfoundland origin) with an iodine value of 135.8 (Burke & Jasperson, 1944) and the blubber fat of a grey Atlantic seal (*Halichoerus grypus*) caught off the coast of Pembroke, with an iodine value of 162.2 (Hilditch & Pathak, 1947). The chief differences observed in the detailed analyses of these two seal oils consisted in variation in the proportions of unsaturated C_{16} acids, unsaturated C_{18} acids, and unsaturated C_{22} acids.

When Prof. A. N. Worden of the University College of Wales, Aberystwyth (who had very kindly put material from the grey Atlantic seal at our disposal earlier), informed us that similar specimens from two common seals (*Phoca vitulina* L.) were to be available, it was felt desirable to undertake similar examination of their blubber and liver lipids in

order to add to the somewhat meagre knowledge of the fatty acids present in the fats of members of this family. Prof. Worden was accordingly good enough to secure for us samples of blubber and of liver from two common seals taken off the Norfolk coast in July 1947, the lipids from which were extracted and examined in our laboratory.

METHODS

The tissues (blubber or liver) were cut into small pieces and extracted exhaustively with hot acetone until no further lipid was removed. The material left after removal of the acetone was taken up in light petroleum (b.p. 40–60°), the solution separated from the water which had also been entrained by the acetone, and the lipids recovered. The liver lipids contained appreciable quantities of phosphatides as well as glycerides, and these were separated by keeping their acetone solutions at 0° for several days, when the phosphatides were precipitated. The component fatty acids of the blubber oils and (so far as the quantities available permitted) of the liver glycerides and phosphatides were determined by the methods used in our earlier work (Hilditch & Pathak, 1947) on the blubber and liver oils of the grey Atlantic seal. These are briefly as follows:

(a) The mixed fatty acids from the oil were first partly resolved by crystallization from acetone and from ether at