Activation of Succinate Dehydrogenase in Heart-Muscle Preparations

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During experiments involving consecutive spectrophotometric assays of succinate-oxidase activity under various conditions, anomalous results were obtained which appeared to be explainable only on the basis of changes taking place in the diluted heart-muscle preparation from which samples were taken from time to time. Confirmation of this explanation was obtained when it was found that succinate-oxidase activity more than doubled when a fresh dilution of the enzyme preparation was kept at 37° for 15 min., and a similar but slower increase in activity was also observed on incubating the diluted enzyme preparation at the temperature of the assays (20°). Further investigations showed that the increase in activity was due to the activation of succinate dehydrogenase. Kearney (1957) described in detail an activation phenomenon observed with the soluble enzyme. Although she noted that a similar activation could be demonstrated with particulate preparations, the possible implications of the work appear to have escaped attention, and the present work was undertaken to investigate in more detail the characteristics of the activation process in such particulate preparations. Certain new observations have been made, and during the work the possible connexion between the activation of succinate dehydrogenase and the requirement for activators of succinate-oxidase preparations in media containing very little inorganic phosphate (Ball & Cooper, 1949; Bonner, 1954) has been explored. A preliminary account of part of this work has already appeared (Thorn, 1961).

METHODS

Heart-muscle preparations. These were made from pig heart according to the general methods of Keilin & Hartree (1947) and Slater (1949), except that the isoelectric precipitates were washed with ice-cold water and finally suspended in buffer at pH 7.6: 0.1M-phosphate, 0.2M-borate (Bonner, 1954) or 0.1M-tris-acetate. These will be referred to as 'phosphate', 'borate' and 'tris' preparations respectively. Occasionally, for comparative purposes, the washed isoelectric precipitate was divided into two portions, and the portions were suspended in different buffers chosen from the three mentioned above. The protein content of preparations was determined as fat-free dry weight (Slater, 1949). Preparations were stored at about 4° and used within 4 days.

Succinate-oxidase activity. This was estimated essentially

as described by Slater & Bonner (1952). The reaction mixture, final vol. 3.0 ml. in a cuvette of 1 cm. light-path, contained: tris-acetate (0.1M), pH 7.6; EDTA (0.25 mM), adjusted to pH 7.6; succinate (25 mM); heart-muscle preparation (about 0.1 mg. of protein for reactions at 20° and half as much for reactions at 37°). The reaction was started by the addition of enzyme. The reaction rates were measured at 230 m μ with a Hilger Uvispek spectrophotometer (H 700.304) fitted with a cuvette carrier through which water from a constant-temperature bath was circulated. By using this device the change in temperature of the cuvette contents during a kinetic run of 5–7 min. was less than 0.1°, and the temperatures of individual runs during a series differed by no more than 0.2°. The temperatures used were between 20° and 21° unless otherwise stated. The specific activity of the enzyme under these conditions, expressed as a change in extinction at 230 m μ /mg. of protein/min., was converted to a Q_{O_2} value by use of the molecular extinction coefficient of fumarate (4.38×10^6 cm.² mole⁻¹) reported by Colpa-Boonstra (1959).

When succinate-oxidase activity was measured manometrically, the same reaction mixture as above was used, except that the final concentration of EDTA was 0.50 mM, and that heart-muscle preparation (about 1 mg. of protein for assays at 37° and about 2 mg. of protein for assays at 20°) was added to the main compartments of the flasks, the reaction being started by tipping succinate from the side arms after temperature equilibration.

Borate buffer (0.1M), pH 7.6, was occasionally used in place of tris-acetate in both spectrophotometric and manometric methods.

Succinate-phenazine-reductase activity. This was used as a measure of succinate-dehydrogenase activity (cf. Giuditta & Singer, 1959), and was estimated manometrically. The reaction mixture was the same as that employed for the manometric estimation of succinate-oxidase activity, except that EDTA was omitted (it was found to be slightly inhibitory), and that KCN (adjusted to pH 7.6, final concn. 10 mM) and phenazine methosulphate (0.5 mg.) were added. The amount of heart-muscle preparation was half that employed for the manometric estimation of succinate-oxidase activity. Buffer, succinate and the appropriate volume of water were pipetted into the main compartments of the flasks. Enzyme preparation, followed immediately by KCN, was then added and the flasks were attached to the manometers. Finally the solution of phenazine methosulphate was pipetted into the side arms, the stoppers were inserted and the flasks placed in the Warburg bath without delay. By this procedure the succinate dehydrogenase was not subjected to possible inhibition by KCN (Tsou, 1951) until after it was protected by succinate, the enzyme was in contact with succinate, a powerful activator (Kearney, 1957), for the longest possible time without catalytic cycling, and the phenazine was

exposed to light for the shortest practicable time (Singer & Kearney, 1957). It was convenient to employ this procedure as a routine rather than to set up a series of flasks with different phenazine concentrations to measure V (Singer, Kearney & Bernath, 1956), and the Q_{O_2} values (after the oxygen consumption had been halved to compensate for H_2O_2 formation) were usually 18–33% greater than the corresponding succinate-oxidase Q_{O_2} values.

Dihydronicotinamide-adenine dinucleotide-oxidase activity. This was estimated spectrophotometrically, essentially as described by Slater (1950). The buffers employed were either phosphate (final concn. 0.05M), pH 7.6, or tris-acetate as used in the estimation of succinate-oxidase activity. EDTA (final concn. 0.25 mM) and about 0.05 mg. of enzyme preparation were used; the final volume was 3.0 ml. The specific activity of the enzyme expressed as a change in extinction at 340 $m\mu$ /mg. of protein/min., was converted to a Q_{O_2} value by use of the molecular extinction coefficient of $NADH_2$ (6.22×10^6 cm.² mole⁻¹).

Dihydronicotinamide-adenine dinucleotide-fumarate reaction. The oxidation of $NADH_2$ by fumarate, in the presence of cyanide, was estimated essentially as described by Slater (1950). The reaction mixture was similar to that employed for the measurement of $NADH_2$ -oxidase activity, but KCN (adjusted to pH 7.6, final concn. 10 mM) and fumarate (final concn. 10 mM) were also added, and the amount of heart-muscle preparation used was 10 times that employed for measurement of $NADH_2$ -oxidase activity.

Fumarate-reductase activity. The reduction of fumarate by FMNH₂ was measured spectrophotometrically at 450 $m\mu$ in Thunberg tubes with attached optical cuvettes of 1 cm. light-path, by slight modifications of the method of Massey & Singer (1957). The modifications were to avoid exposing the enzyme either to reducing conditions or to prolonged and uncontrolled fluctuations of temperature during the preliminary preparation of the reaction mixture. The reaction medium finally contained: phosphate (0.05M), pH 7.6; fumarate (33 mM); FMNH₂ (0.2–0.8 mM); heart-muscle preparation (about 1 mg.); the final volume was 3.0 ml. All components of the reaction mixture except the enzyme were added to a Thunberg cuvette in a volume of 2.6 ml., an empty stopper was inserted and the procedure described by Massey & Singer (1957) for converting the FMN almost completely into FMNH₂ was followed. The empty stopper was then quickly exchanged for a similar stopper containing 0.4 ml. of suitably diluted and pretreated heart-muscle preparation, and the tube was re-evacuated and refilled with O_2 -free N_2 four times. The contents of the tube were warmed in a water bath at 20° for 10 min., the enzyme was tipped, and the extinction at 450 $m\mu$ determined as a function of time. With this technique it was found, before tipping the enzyme, that no spontaneous reoxidation of FMNH₂ took place owing to incomplete anaerobiosis. The specific activity of the enzyme was expressed as μ moles of fumarate reduced (or of FMNH₂ oxidized)/mg. of protein/hr., by employing the difference in the molecular extinction coefficients of FMN in the oxidized and reduced forms at 450 $m\mu$ (11.3×10^6 cm.² mole⁻¹). The enzyme activity was determined at several concentrations of FMNH₂, and V was calculated from double reciprocal plots of activity against FMNH₂ concentration (Lineweaver & Burk, 1934).

General procedure for following activation. Standard dilutions of heart-muscle preparations (0.8–1.2 mg. of

protein/ml.) were prepared by pipetting a suitable volume of enzyme into about 15 ml. of the required buffer at 0° in a 25 ml. glass-stoppered volumetric flask surrounded with ice. The mixture was swirled gently and made up to the mark with more buffer at 0°. This diluted mixture was kept at 0° throughout an experiment, and the succinate-oxidase activity remained constant or increased by only a few per cent during several hours under these conditions. A sample (2–3 ml.) of the dilution was pipetted into a test tube which was then covered and placed in a water bath at the desired temperature at a noted time. At appropriate times during this incubation a 0.1 ml. sample was withdrawn and added to the reaction mixture in a prepared cuvette for the estimation of succinate-oxidase activity. The time taken for the few millilitres of standard dilution at 0° to attain the required incubation temperature was ignored. When it was required to follow the course of the very rapid activation which took place at 32° (see Fig. 1), this source of error was reduced by using the following procedure. Each experimental point was obtained by diluting the appropriate volume of concentrated heart-muscle preparation at 0° to 2.5 ml. in a test tube containing the required volume of buffer previously warmed to 32°, and the length of the incubation time was estimated to the nearest second, from the moment of mixing the heart-muscle preparation with the warmed buffer to the moment of mixing a sample of the incubated suspension with the reaction mixture for estimating succinate-oxidase activity.

Buffers. Buffers for enzyme-activity measurements were made up at double the final concentration required in the reaction medium, and were at such a pH that on dilution the resulting pH was 7.6 ± 0.02 at the temperature at which the assay was to be performed. Phosphate buffers were mixtures of KH_2PO_4 and Na_2HPO_4 , 0.2M-borate buffer was similar to that used by Bonner (1954) but the amount of borax solution added was adjusted to the requirement mentioned above, and tris-acetate buffers were made from tris (Sigma 121 grade) and acetic acid.

Other reagents. AnalaR-grade chemicals were used. $NADH_2$ was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and FMN from British Drug Houses Ltd., Poole, Dorset. Phenazine methosulphate was prepared by the Singer & Kearney (1957) modification of the method of Dickens & McIlwain (1938). Distilled water, subsequently passed through a mixed-bed de-ionizing column (Amberlite MB1), was used in making up all solutions.

RESULTS

Characteristics of activation of succinate dehydrogenase in heart-muscle preparations

Evidence that the observed activation of succinate oxidase is due to the activation of succinate dehydrogenase. Succinate-oxidase and succinate-phenazine-reductase activities of diluted heart-muscle preparations were determined before and after warming at 37° (Table 1). With the three methods of assay, the activated activities of the two types of preparation were very similar; the increases in activity recorded in the manometric assays in each type of preparation were also similar, suggesting that the increases in succinate-oxidase activity

were caused by the increase in succinate-dehydrogenase activity. The manometric assays show a smaller increase in activity than the spectrophotometric method because some activation of the samples kept at 0° took place during the time between pipetting the enzyme into the flasks and the time when measurements were begun, and no such delay intervenes in the spectrophotometric method. The tris preparation had lower initial activities than the phosphate preparation, and a possible reason for this is suggested in the Discussion section.

Further suggestive evidence that the increase in succinate-oxidase activity was due only to an increase in succinate-dehydrogenase activity would be the finding that the NADH₂-oxidase activity of

the preparation is unaffected by the activation procedure, but that there is an increase in other enzymic activities of the preparation in which succinate dehydrogenase participates (e.g. the NADH₂-fumarate reaction, fumarate-reductase activity). The results given in Table 2 show that NADH₂-oxidase activity tended to fall to a greater or lesser extent while succinate-oxidase activity increased. The fall in NADH₂-oxidase activity might be expected in view of the well-known instability of the enzyme system in very dilute suspensions (Slater, 1950). A slight increase in rate of the NADH₂-fumarate reaction, and a definite increase in fumarate-reductase activity, occurred simultaneously with an increase in succinate-oxidase activity. After incubation at 37° the

Table 1. *Succinate-oxidase and succinate-phenazine-reductase activities of heart-muscle preparations before and after incubation at 37°*

Heart-muscle preparation, standard dilution for spectrophotometric assays and 8.93 mg. of protein/ml. for manometric assays, in 0.1M-phosphate, pH 7.6, at 0°, was pretreated before assay as shown. Enzyme activities were measured at 20° in 0.1M-tris-acetate, pH 7.6, as described in the Methods section, except that the heart-muscle preparation in the phenazine assay was treated during the equilibration period in the same way as that in the manometric oxidase assay, by placing it in the side arms of the flasks, and all other reagents in the main compartments. The percentage increases in activity after warming are shown in parentheses.

Type of preparation*	Pretreatment before assay	Succinate-phenazine-reductase activity (Q_{O_2})	Succinate-oxidase activity (Q_{O_2})	
			Manometric	Spectrophotometric
Tris	Kept at 0°	189	158	75
	37°/20 min.	316 (+67%)	269 (+70%)	261 (+248%)
Phosphate	Kept at 0°	267	226	180
	37°/20 min.	339 (+27%)	288 (+27%)	269 (+50%)

* The tris and phosphate preparations were made simultaneously from the same batch of heart muscle.

Table 2. *Succinate-oxidase, dihydronicotinamide-adenine dinucleotide-oxidase, dihydronicotinamide-adenine dinucleotide-fumarate-reductase and fumarate-reductase activities of heart-muscle preparations before and after incubation at 37°*

Standard dilutions of tris preparations (but dilutions of about 3.0 mg. of protein/ml. in fumarate-reductase assays) in 0.1M-phosphate, pH 7.6, at 0°, were pretreated before assay as shown. Enzyme activities were measured as described in the Methods section; the buffer for assays was 0.05M-phosphate, except for succinate oxidase in Expt. 83S when 0.1M-tris-acetate was used; the temperature was 25° in Expt. 71S and 74S, and 20° in Expt. 83S. Fumarate-reductase activities are expressed as *V* values. Percentage changes in activity after warming are shown in parentheses.

Preparation	Expt.	Pretreatment before assay	Succinate-oxidase activity		NADH ₂ -oxidase activity		NADH ₂ -fumarate-reductase activity (μmole/mg./hr.)	Fumarate-reductase activity (μmole/mg./hr.)
			(Q_{O_2})	(μmoles/mg./hr.)	(Q_{O_2})	(μmoles/mg./hr.)		
52T	71S	Kept at 0°	100	8.9	603	53.8	—	—
		37°/30 min.	344	30.7 (+244%)	426	38.0 (-29.5%)	—	—
	74S	Kept at 0°	92	8.2	562	50.2	0.434	—
		37°/30 min.	339	30.2 (+269%)	554	49.5 (-1.3%)	0.590 (+36%)	—
54T	83S	Kept at 0°	46.4	4.14	—	—	—	0.478
		37°/30 min.	189	16.9 (+308%)	—	—	—	0.771 (+61.4%)

fumarate-reductase activity of the preparation was about 5% of the succinate-oxidase activity, in agreement with the findings of Giuditta & Singer (1959).

These experiments, taken together, leave little doubt that the observed activation of succinate-oxidase activity was due to an activation of succinate dehydrogenase.

Kinetics of activation in 0.1M-phosphate. The course of activation of succinate dehydrogenase in a tris preparation diluted in 0.1M-phosphate, pH 7.6, as revealed by changes in succinate-oxidase activity, is shown in Fig. 1. In agreement with Kearney (1957), the activation was negligible over a period of hours at 0° (see Fig. 2), but was very fast at 37°. The activation process obeys first-order kinetics, the half-times being about 60, 20 and 6 min. respectively at 20°, 26° and 32°. From the results shown in Fig. 1 the first-order rate constants were calculated, and, by means of an Arrhenius plot, the activation energy for the activation process was found to be 34.7 kcal., in good agreement with Kearney's reported value (36.5 kcal.). These properties suggest that the process studied in the present work was the same as that discovered by Kearney.

Variation of the concentration of heart-muscle preparation (in 0.1M-phosphate, pH 7.6) between 0.8 and 8.0 mg. of protein/ml. had no significant effect on the course of activation at 20°. The rate of activation at 0.4 mg./ml. appeared to be slightly slower, and this was attributed to simultaneous inhibitory changes taking place at the greater dilution. Concentrated heart-muscle preparation (25–30 mg./ml.), however, underwent activation

much more slowly. Thus, in one experiment, the degree of activation of a standard dilution incubated at 20° for 53 min. was 49%, whereas that of the concentrated preparation (originally made up in 0.1M-phosphate) incubated at 20° for 69 min. was only 8%.

Stability of succinate-oxidase activity in 0.1M-phosphate. The experiments (Fig. 1) show that, after dilution of heart-muscle preparation in 0.1M-phosphate, the succinate-oxidase activity did not become stable until a certain time had elapsed, depending on the temperature. The stability of succinate-oxidase activity at 0° is shown in Fig. 2. Whatever the degree of activation, the succinate-oxidase activity changed by only a few per cent during 2–3 hr. The initial rise for the partially activated sample was due to the continuation of activation during the time taken for the enzyme suspension to cool from 37° to 0°, after the first sample had been removed for the determination of activity. The activation on warming in phosphate does not appear to be reversible on cooling; the slight fall in activity of the fully activated enzyme was probably due to a simultaneous inhibitory process. Kearney (1957) was unable to reverse the activation of the soluble enzyme.

Activation in non-phosphate media. Kearney (1957) reported that, for activation to take place, it was necessary to incubate succinate dehydrogenase with either a substrate (succinate or fumarate) or a competitive inhibitor, e.g. phosphate (Slater & Bonner, 1952) or malonate, i.e. with a substance that combines with the active centre of the enzyme. If this occurred in particulate preparations, the low succinate-oxidase activities observed by

Fig. 1. Change in succinate-oxidase activity on incubating diluted heart-muscle preparation in phosphate at various temperatures. Standard dilution of tris preparation in 0.1M-phosphate, pH 7.6, at 0°, was incubated and sampled as described in the Methods section. Succinate-oxidase activities were determined spectrophotometrically as described in the Methods section. ○, 20.5°; △, 26.3°; □, 32.1°; ●, 37.0°.

Fig. 2. Change in succinate-oxidase activity on keeping dilute heart-muscle preparation, in various stages of activation, at 0°. Standard dilution of tris preparation in 0.1M-phosphate, pH 7.6, at 0°, was assayed spectrophotometrically as described in the Methods section. ○, Sample of dilution kept at 0°; △, sample of dilution warmed to 37° for 5 min. and then kept at 0°; □, sample of dilution warmed to 37° for 30 min. and then kept at 0°.

Bonner (1954) in preparations suspended, and assayed manometrically at 37°, in borate buffer, might be explained. It was therefore decided to study changes in succinate-oxidase activity on incubation of dilute suspensions of heart-muscle preparation in non-phosphate media.

Fig. 3 shows that activation in tris-acetate at 37° was almost as rapid and complete as that in phosphate buffer, but that prolonged incubation led to progressive losses in activity, presumably owing to an inhibitory process unconnected with activation. This is also seen in borate buffer, in which the effect of the activation was opposed by a much more rapid simultaneous inactivation. The slight susceptibility to loss of activity in tris-acetate compared with phosphate, and the greater susceptibility in borate, agrees with general observations on the keeping qualities of heart-muscle preparations made in phosphate, tris-acetate and borate buffers, and stored at about 4° (M. B. Thorn, unpublished work). However, EDTA added to the borate incubation medium largely suppressed the loss of activity, and activation proceeded approximately to the level attained in tris-acetate.

The final degree of activation reached in phosphate was independent of the temperature (Fig. 1), but only partial activation took place in tris-

Fig. 3. Change in succinate-oxidase activity on incubating, at 37°, standard dilutions of different heart-muscle preparations in different buffers at pH 7.6. Enzyme activities were determined spectrophotometrically as described in the Methods section, and are given as percentages of total activation, where the unactivated activity is 0% and the activity after warming at 37° in phosphate for 30 min. is 100%. ○, Tris preparation diluted in 0.1 M-phosphate; △, tris preparation diluted in 0.1 M-tris-acetate; □, borate preparation diluted in 0.1 M-borate; ●, borate preparation diluted in 0.1 M-borate containing EDTA (0.5 mM).

acetate at 20° and thereafter activity declined, as with prolonged incubation at 37°. The failure to reach full activity at 20° might have been solely because of an opposing inhibitory process. To test this, an experiment (Fig. 4) was performed in which the actual course of activation (lower line) was compared with the change in potential activity (upper line). The fact that the two curves did not converge, but eventually ran in parallel, indicates that the failure to reach complete activation in tris-acetate at 20° was not primarily due to simultaneous inhibition. It could, however, be explained by postulating that conversion of the enzyme into the activated form, in tris-acetate, was a reversible process with a temperature-dependent equilibrium.

Reversibility of activation of succinate dehydrogenase in tris-acetate. This was demonstrated by the finding that fully activated preparation on cooling at 0° slowly lost activity that was then rapidly regained on reincubation at 37°. The approach to a partially activated equilibrium mixture at 20°, both from the unactivated and the fully activated condition, is shown in Fig. 5. Both samples, on being transferred to 37°, became almost completely activated. A similar experiment was performed at 20°, 25° and 30°, with another preparation, in an attempt to establish the positions of equilibrium at a variety of temperatures, to permit calculation of thermodynamic quantities associated with the conversion of the unactivated form of succinate dehydrogenase into the activated form. The calcula-

Fig. 4. Course of activation, and potential degree of activation, of heart-muscle preparation incubated in tris-acetate at 20°. Tris preparation was diluted to 2.16 mg. of protein/ml. in 0.1 M-tris-acetate, pH 7.6, at 0°, and about 10 ml. of this dilution was incubated at 20°. At various times, a 1 ml. sample was removed and mixed with 1 ml. of 0.1 M-phosphate, pH 7.6, at 0°, and the succinate-oxidase activity was determined spectrophotometrically, as described in the Methods section, both immediately (○) and after further incubation at 37° (△) for 20 min. (for 30 min. with the zero-time sample).

tion of equilibrium constants is uncertain because: (a) the time taken to reach equilibrium is so long that simultaneous inhibition is liable to be a complication (cf. Fig. 4), though this was not a serious objection in Fig. 5 as shown by the almost complete recovery of activity at 37°; (b) there is doubt about the activity of the unactivated form of the enzyme, which is probably not a single species but a mixture of forms with high and low activities, and the so-called 'low-activity' form may even be completely inactive. If it is assumed that the 'low-activity' form is inactive, the relative concentrations of the activated and unactivated forms, and

hence the equilibrium constant K (% activated form/% unactivated form), may be calculated (Table 3). A plot of $\log K$ against the reciprocal of the absolute temperature yielded a straight line, from the slope of which ΔH , the change in heat content on conversion of the unactivated into the activated form, was found to be 20 kcal./mole. Table 3 also shows ΔF and ΔS at each temperature, from calculations analogous to those (cf. Stearn, 1949) applied to data on the reversible denaturation of trypsin (Anson & Mirsky, 1934). The value for ΔS is high, but only about one-third of that associated with reversible denaturation of trypsin.

Fig. 5. Approach of succinate-oxidase activity of dilute heart-muscle preparation to the equilibrium degree of activation at 20° in tris-acetate. Standard dilution of tris preparation in 0.1 M-tris acetate, pH 7.6, at 0°, was assayed spectrophotometrically as described in the Methods section. ○, Course of activation on incubating a sample at 20°; the sample was transferred to 37° at the time indicated by the arrow. △, Course of deactivation on incubating a sample, previously warmed at 37° for 20 min., at 20°; the sample was returned to 37° at the time indicated by the arrow.

Consequences of the activation of succinate dehydrogenase

Comparison of different assay methods, and of activities at different temperatures. Table 4 shows how misleading results for succinate-oxidase activity might be obtained with manometric and spectrophotometric methods at different temperatures if the activation phenomenon is ignored. Only in the manometric method at 37° could activation be ignored, since the temperature and duration of the equilibration period were sufficient to allow for adequate activation. Manometric and spectrophotometric methods were in good agreement at 20° when prior precautions were taken to activate succinate dehydrogenase. The error involved in ignoring the activation process at 20° was less in the manometric method than in the spectrophotometric method, because partial activation took place during the equilibration period [the discrepancy between manometric and spectrophotometric methods of assay of succinate-oxidase activity at 25° (Holton & Colpa-Boonstra, 1960) is probably due to the different degrees of activation

Table 3. *Equilibrium constants, and free energy and entropy changes, for the conversion of unactivated into activated succinate dehydrogenase*

The succinate-oxidase activities in the second column (except the first value, see below) are the averages of the activities of two samples of enzyme approaching equilibrium activity in 0.1 M-tris acetate, pH 7.6, one after warming from 0°, the other after cooling from 37° (cf. Fig. 5). The maximum activity to which these values were referred was the activity observed after warming the same preparation in 0.1 M-phosphate, pH 7.6, for 30 min. at 37°. In column 4, $\Delta F = -RT \ln K$, and in column 5, $\Delta S = (\Delta H - \Delta F)/T$.

Temperature (°K)	Succinate-oxidase activity at equilibrium (% of maximum)	K (% activated /% unactivated)	ΔF (cal./mole)	ΔS (cal./mole/degree)
277	22.0*	0.282	697	69.7
293	68.6	2.19	-456	69.8
298	77.8	3.51	-743	69.6
303	86.5	6.41	-1120	69.7

* The value at 277°K is the initial unactivated activity of the tris preparation immediately after dilution in tris-acetate at 0°: it is assumed that equilibrium between unactivated and activated forms had already been reached during storage of the enzyme for 3 days at an average temperature of 4°.

in the two methods]. The unexpected difference in succinate-oxidase activities of the activated preparation, measured at 37° by the two methods, is real. The spectrophotometric value was confirmed within 3% with a separate dilution of enzyme. The temperature coefficient for the ultraviolet absorption of fumarate (Massey, 1953) is too small to account for the higher Q_{O_2} calculated for the spectrophotometric method. The agreement between the two manometric results at 37° may be coincidental and misleading, because the sample kept at 0° may have failed to reach complete activation during equilibration in tris-acetate and EDTA in the Warburg flask, and the warmed sample may have lost some activity owing to its longer incubation at 37°.

The Arrhenius activation energy of the succinate-oxidase reaction calculated from the spectrophotometric results at 20° and 37° with the activated preparation is 13.0 kcal.; the value calculated from the corresponding manometric results is 10.7 kcal.

These results support the previously reported value of 11.25 kcal. (Thorn, 1951).

Effect of activation on K_m . A previous value for K_m of the succinate-oxidase system (0.48 mM at 25°), determined spectrophotometrically by Slater & Bonner (1952), was obtained before the activation of succinate dehydrogenase was known. Other values (Thorn, 1953) of K_m for similar preparations, determined absorptiometrically and with ferricyanide as electron acceptor, were not inconsistent with that given by Slater & Bonner; in a series of preparations a spread of results between 0.25 and 0.53 mM at about 20° was noted and unexplained. It was decided to determine K_m values, taking into account the activation of succinate dehydrogenase, because this phenomenon might have been responsible for previous inconsistencies.

Results of determining K_m under various conditions are given in Table 5. If the higher values obtained in a phosphate medium were neglected, K_m for activated preparations was between 0.15

Table 4. Comparison of manometric and spectrophotometric assays of succinate-oxidase activity at 20° and 37° with activated and unactivated heart-muscle preparation

Heart-muscle preparation, standard dilution for spectrophotometric assays and 5.0 mg. of protein/ml. for manometric assays, in 0.1M-phosphate, pH 7.6, at 0°, was pretreated before assay as shown. Activities were determined as described in the Methods section, in 0.1M-tris-acetate, pH 7.6 (different buffers used at 20° and 37°).

Pretreatment before assay	Succinate-oxidase activity (Q_{O_2})			
	At 20°		At 37°	
	Manometric	Spectrophotometric	Manometric	Spectrophotometric
Kept at 0°	112	66	616	562*
37°/30 min.	227	226	620	743

* Reaction accelerating: this is the average rate between 3 and 6 min.

Table 5. K_m of succinate oxidase under various conditions at 20–21°

Heart-muscle preparations designated with 'T' in first column were tris preparations and the remainder were phosphate preparations. Activation medium in second column: P, 0.1M-phosphate, pH 7.6, at 37° for 30 min.; T, 0.1M-tris-acetate, pH 7.6, at 37° for 20 min. Assay medium in third column: P, 0.15M-phosphate, pH 7.6; T, 0.1M-tris-acetate, pH 7.6. Values of K_m and V were calculated from double reciprocal plots (Lineweaver & Burk, 1934) of activity against succinate concentration.

Prep.	Activation medium	Assay medium	Activated preparation		Unactivated preparation	
			V (Q_{O_2})	K_m (mM)	V (Q_{O_2})	K_m (mM)
45T	P	T	232	0.20	49.6	0.15
46T	P	T	290	0.20	—	—
		P	265	0.38*	—	—
37	T	T	154	0.15	—	—
		P	150	0.40*	—	—
3P	P	T	179	0.20	—	—
10P	P	T	216	0.16	120	0.15
Purified soluble succinate dehydrogenase at 15° (Kearney, 1957)			—	0.31	—	0.18

* Corrected for competitive inhibition of phosphate in assay medium ($K_i = 0.1M$) (Slater & Bonner, 1952).

Table 6. *Effect on the succinate-oxidase activity of the concentration of heart-muscle preparation in borate buffer during incubation at 37°*

Borate preparation was diluted in buffer, pH 7·6, at 0°, and incubated at 37° as shown. Succinate-oxidase activity was determined spectrophotometrically at 21·5° as described in the Methods section; the buffer was 0·1M-borate, pH 7·6, and 0·108 mg. of heart-muscle preparation was used in each determination.

Conditions during incubation at 37°			Succinate-oxidase activity (Q_{O_2})
Medium	Concn. of heart-muscle preparation (mg. of protein/ml.)	Time (min.)	
0·1M-Phosphate	1·08	30	149
0·2M-Borate + 0·5 mM-EDTA	1·08	26	144
0·2M-Borate	1·08	15·5*	123
0·2M-Borate	0·36	15·5*	101
0·2M-Borate	0·216	15·5*	72·5

* When the time of incubation was extended to 26 min., as in the presence of EDTA, the succinate-oxidase activity fell (see Fig. 3).

and 0·2 mM, and for unactivated preparations was 0·15 mM. Preparation 45T, which underwent a more than fourfold increase in activity, showed an increase in K_m of only 33 % on activation. The K_m at 15° of the soluble preparation reported by Kearney (1957) also underwent an increase of only 72% on activation. These results are considered in the Discussion section.

Possible relation of the activation of succinate dehydrogenase to the need of succinate oxidase for activators in low-phosphate media. It was shown above that, on warming dilute heart-muscle preparation in borate buffer at 37°, the succinate-oxidase activity was susceptible to inhibitory processes as well as to activation, but that inclusion of EDTA in the medium appeared to protect the enzyme from inhibition. The conditions of incubation in this type of experiment are similar to the conditions in the main compartment of a Warburg flask during the equilibration period before tipping the substrate. Moreover, the experiment described in Table 6 shows that the succinate-oxidase activity after incubation in borate at 37° in the absence of EDTA depended on the concentration of heart-muscle preparation, suggesting that susceptibility to loss of activity increases with increasing dilution. These results are significant in view of Bonner's (1954) finding that in a manometric experiment the lowering of activity of succinate oxidase in the absence of activators was greatest at the lowest enzyme concentrations.

Such considerations led to the type of experiment illustrated in Fig. 6. This shows the effect of enzyme concentration on succinate-oxidase activity, determined manometrically at 37° in 0·1M-borate buffer, in the presence and absence of EDTA. The general shape of the lower curve in the absence of EDTA is similar to a corresponding experiment by Bonner (1954), except that the straight portion of

Fig. 6. Effect of concentration of borate preparation on succinate-oxidase activity, determined manometrically at 37° in 0·1M-borate, pH 7·4, in the presence and absence of EDTA, and of exposing the enzyme in the absence of EDTA to different conditions during the equilibration period. O, Enzyme in main compartment of flask with 0·5 mM-EDTA; succinate added from side arm after equilibration; □, enzyme in main compartment of flask without EDTA; succinate added from side arm after equilibration; Δ, no EDTA, enzyme in side arm with succinate during equilibration period.

the curve intercepts the abscissa at about 0·25 mg. of heart-muscle preparation, whereas the intercept in Bonner's experiment occurred at about 1·9 mg. of preparation. In Fig. 6 the intercept was diminished by altering the conditions undergone by the heart-muscle preparation during equilibration, but

the slope was unaffected. The upper curve in the absence of EDTA was obtained by placing the preparation in the side arms of the flasks together with a small volume of succinate solution, to expose the succinate dehydrogenase to an activator (in Kearney's sense rather than Bonner's sense) during equilibration. Although this technique was not successful in completely eliminating the intercept observed in the absence of EDTA, the lack of linearity in this type of experiment can probably be explained either by incomplete activation of succinate dehydrogenase or by an inhibitory process occurring simultaneously with the activation, or by both, during the equilibration at 37° on the Warburg bath.

Even if experimental conditions could have been devised to eliminate the intercept, the slope of the lines in the absence of EDTA is about 7% less than in its presence. Although Bonner (1954) stated that the slope of the straight portion of the curve was the same as the slope of the line in the presence of an activator, measurements of his published Figure suggest that the slope was about 5% less. Stimulation of activation of succinate dehydrogenase, or protection from inhibition, during the equilibration period of a manometric experiment, is therefore only one effect of EDTA on succinate oxidase. A second effect is shown in Fig. 7, where the succinate-oxidase activity of a preparation, fully activated in Kearney's sense by prior incubation

Fig. 7. Rates of fumarate formation, determined spectrophotometrically at 230 $m\mu$, by heart-muscle preparation in the presence and absence of EDTA. Standard dilution of tris preparation in 0.1M-tris-acetate, pH 7.6, at 0°, was activated before each reaction by mixing 2.0 ml. with 0.2 ml. of 0.375M-succinate, and warming the mixture at 37° for 10 min. ○, Medium containing 0.25 mM-EDTA; △, medium without EDTA, but EDTA added to give a final concn. of 0.25 mM at the time indicated by the arrow, and readings of extinction for first period corrected to a volume of 3.0 ml. The positions of the two lines on the scale of extinction, relative to one another, have been adjusted for presentation.

with succinate, but in a reaction medium containing no activators (in Bonner's sense), abruptly increased on the addition of EDTA. It had previously been shown that full activation occurred on incubation with succinate under the conditions specified.

DISCUSSION

Kearney (1957) reported that the activation of soluble succinate dehydrogenase was accompanied by a change in its absorption spectrum, and published a difference spectrum of activated enzyme minus unactivated enzyme. F. A. Holton & M. B. Thorn (unpublished work) used a highly sensitive recording spectrophotometer to observe changes in extinction of dilute Keilin & Hartree (1947) preparation undergoing activation. The observed changes were explained by a decrease in light-scattering of the suspension, together with a net reduction of flavoprotein-like pigment, and the changes expected on the basis of Kearney's (1957) difference spectrum either were not present or were obscured. Nevertheless, the increases in enzymic activity of those parts of the respiratory chain involving succinate dehydrogenase (succinate-phenazine reductase, NADH₂-fumarate reductase and FMNH₂-fumarate reductase), and the lack of effect on NADH₂ oxidase, suggest that the increases in succinate-oxidase activity studied in this work are the result of activation of succinate dehydrogenase, and the similarity of the kinetics of activation in 0.1M-phosphate strongly supports the conclusion that the phenomenon observed in the present work is the same as that discovered by Kearney (1957).

The main differences between Kearney's (1957) results and those reported here are: first, activation of the soluble enzyme appears to take place only on incubating the enzyme with a compound that combines with the active centre, whereas with the Keilin & Hartree (1947) preparation it is difficult to prevent activation taking place in any medium (although it may be incomplete, and is negligible at 0° for some hours); secondly, the process was irreversible with the soluble enzyme, but was reversible under certain conditions with the Keilin & Hartree (1947) preparation.

To describe the activation phenomenon the following simple tentative scheme is proposed:

where E_U , E_A and E_I represent respectively unactivated, activated and irreversibly inactivated forms of succinate dehydrogenase. The initial equilibrium between unactivated and activated

forms is governed by the temperature and can be investigated in a medium in which the enzyme is largely protected from inactivation (e.g. the particulate enzyme in tris-acetate) and which is free of compounds (represented by C in the above scheme) that combine with the enzyme either as substrate or competitive inhibitors (to give a E_4C complex). The fact that the reversibility of the activation is not apparent in phosphate would be explained by the displacement of the activation equilibrium by combination of the activated enzyme with phosphate. It is, however, not clear why Kearney (1957) failed to detect a reversal of activation by dialysis of the soluble preparation after activation in the presence of malonate, unless the dialysis was insufficiently prolonged to remove all of the activator. For the particulate preparation used in the present work, the conversion of the enzyme into the inactivated form may be due to an autoxidation of the essential thiol groups of the enzyme; Thorn (1958) showed that competitive inhibitors or anaerobic conditions protected the enzyme from loss of activity in a borate medium. Although the inactivation reaction may not be the same with the soluble enzyme (Singer, Kearney & Massey, 1957), competitive inhibitors (or substrates) are likely to act as protectors. The activators employed by Kearney (1957) could therefore have acted as protectors of the activated form of the enzyme rather than as true activators; probably activation in their absence could not be demonstrated owing to the greater susceptibility of the soluble enzyme to inactivation. In view of the present studies on borate buffer as an activation medium, in which succinate dehydrogenase in the particulate preparation is relatively unstable compared with its behaviour in tris-acetate and phosphate buffers, simultaneous activation and inhibition could mutually obscure one another during the equilibration period in a manometric method. This tentative scheme therefore provides a basis for interpreting the observations both with the soluble and the particulate preparations.

The scheme also implies that the unactivated form of the enzyme is not catalytically active, and does not undergo the same inactivation reaction as the activated form. The consistency of the thermodynamic calculations (Table 3) supports this suggestion, but does not rule out the possibility that the unactivated form may be about 10% as active as the activated form. If the unactivated form has catalytic activity, the suggested scheme requires amplification to show the reversible combination of E_σ with C to give a $E_\sigma C$ complex. The fact that reversal of activation does not take place in phosphate might then be represented by an additional activation reaction, i.e. an irreversible conversion of $E_\sigma C$ into E_4C .

The chemical basis of the activation phenomenon is not understood, but Singer *et al.* (1957) suggested that it represents a 'major change in the configuration of the enzyme'. The present work throws no new light on this problem, but the above suggestion is supported indirectly by the relatively high entropy change on conversion of the unactivated form of the enzyme into the activated form. Massey (1958) described another phenomenon which takes place on incubation of soluble succinate dehydrogenase at 25°: the iron present in the enzyme becomes more accessible to chelators, and this process is accompanied by an increase in turbidity, representing denaturation. The results of Kearney (1957) and Massey (1958), taken together, suggest that the conversion of unactivated into activated enzyme involves a change in configuration of the enzyme protein that also exposes the iron atoms, and that the soluble enzyme in this activated form then rapidly changes into the inactivated (denatured) form. Since Massey (1958) was unable to find evidence of participation of the iron atoms in catalytic activity, it must be presumed that changes in protein configuration in relation to the active centre are the important factor in activation.

Although Singer *et al.* (1957) considered that the activation was not a 'reactivation of previously inactivated enzyme', the present demonstration of the reversibility of the process renders this possibility quite feasible, and Fig. 8 supports this contention. The activated activities of tris and phosphate preparations, made from the same batch of heart muscle, were similar and fairly constant for several days. The activities before activation, immediately after making the preparations, were also similar, but that of the phosphate preparation then rose slowly, whereas that of the tris preparation fell and became steady. The rise with the phosphate preparation can be explained by a slow displacement of the activation equilibrium by combination of the activated form of the enzyme with phosphate, whereas the fall with the tris preparation can be explained by the approach to equilibrium between the unactivated and activated forms of the enzyme at about 4°. This fall could have begun during the preparation of the enzymes, since much of the process was carried out at about 0°, and the enzyme was exposed to low phosphate concentrations (e.g. when washing the isoelectric precipitate with water at 0°). It is likely that in the tissues the enzyme is present in the activated form, and that at least partial conversion into the unactivated form takes place during preparation.

Activation involving a fourfold increase in activity was accompanied by an increase in K_m of only 33%. Although in the present experiments a 33% change in K_m may not be a significant

Fig. 8. Daily variation of unactivated and activated succinate-oxidase activity of heart-muscle preparations stored at about 4°. Tris and phosphate preparations were made simultaneously from the same batch of heart muscle. Succinate-oxidase activities of standard dilutions in 0.1M-phosphate, at 0°, were determined spectrophotometrically as described in the Methods section, before (unactivated) and after (activated) warming the diluted enzyme at 37° for 30 min. ○, Phosphate preparation, unactivated; ●, phosphate preparation, activated; □, tris-acetate preparation, unactivated; ■, tris-acetate preparation, activated.

difference, Kearney (1957) reported a larger increase (72%). From the relative values of k_{+1} , k_{-1} and k'_{+2} reported by Slater & Bonner (1952), and confirmed by Thorn (1953), a fourfold increase in activity would be expected to lead to an increase in K_m [that is, $(k_{-1} + k'_{+2})/k_{+1}$] of about threefold (assuming that the reported values of the rate constants apply to the fully activated state). The relatively small observed increase in K_m suggests that activation involves an increase in k_{+1} , or a decrease in k_{-1} , or both, compensating partly for the large increase in k'_{+2} .

The investigation of the effect of EDTA on succinate-oxidase activity in borate buffer has shown that low activities obtained manometrically in the absence of phosphate, histidine or EDTA (Ball & Cooper, 1949; Bonner, 1954) have no simple explanation. The effect is a complex one, resulting partly from concentration-dependent effects on succinate dehydrogenase itself (failure of complete activation, or an inhibitory process, or both, in the absence of EDTA) and partly from a requirement of the succinate-oxidase system for activators. These two types of effect have been distinguished in the present work. Ball & Cooper (1949) showed that inhibitory changes took place during the equilibration period on the Warburg bath, although their interpretation of the inhibitory changes is considered to be incorrect (Thorn, 1958; and the present work). The involvement of succinate dehydrogenase in the phenomena observed with

low-phosphate preparations was not suspected as long as methylene blue was used in the estimation of 'succinate-dehydrogenase activity'. Bonner's (1954) conclusions about the point of action of the activating effect of histidine and EDTA in the remainder of the succinate-oxidase system are unaffected by the present work, but the magnitude of the effects he described was probably increased by the unsuspected complication of the activation of succinate dehydrogenase.

SUMMARY

1. Keilin & Hartree (1947) heart-muscle preparations, diluted in phosphate buffer, showed increases in succinate-oxidase activity of up to more than fourfold on incubation at 37°. This increase in activity was accompanied by an increase in succinate-phenazine-reductase activity and smaller increases in rate of the NADH_2 -fumarate and FMNH_2 -fumarate reactions, but the NADH_2 -oxidase activity was unaffected or inhibited. It was concluded that the activation of succinate-oxidase activity was caused by the activation of succinate dehydrogenase (Kearney, 1957).

2. The kinetics of activation of succinate-oxidase activity of heart-muscle preparation, diluted in phosphate, were similar to those of soluble succinate dehydrogenase, as described by Kearney (1957).

3. The activation of succinate-oxidase activity also took place in tris-acetate and borate buffers, but simultaneous inhibition tended to occur on prolonged incubation. This inhibition was greater in borate, especially at increasing dilutions of enzyme, but EDTA had a protective effect.

4. The activation of succinate dehydrogenase in heart-muscle preparations diluted in tris-acetate was reversible, and the equilibrium degree of activation depended on the temperature. When it was assumed that one form of the enzyme is catalytically inactive, calculations from the equilibrium data yielded consistent values for the changes in heat content and entropy for the conversion of the inactive into the active form. These values were: $\Delta H = 20 \text{ kcal./mole}$; $\Delta S = 69.7 \text{ cal./mole/degree}$.

5. The K_m of succinate oxidase, determined in tris-acetate at 20°, was 0.15 mM for unactivated preparations, and 0.15–0.2 mM for activated preparations.

6. Low succinate-oxidase activities observed in manometric experiments in borate buffer are the result of: (a) a failure of activation of succinate dehydrogenase; (b) a specific requirement of succinate oxidase for activators (Bonner, 1954). Phosphate and EDTA appear to act as protectors in the first case and as activators in the second.

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The Active Site and Mechanism of Action of Bovine Pancreatic Ribonuclease

3. THE pH-DEPENDENCE OF THE KINETIC PARAMETERS FOR THE HYDROLYSIS OF CYTIDINE 2',3'-PHOSPHATE*

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Only limited data on the variation of the activity of bovine pancreatic ribonuclease with pH have been reported in the literature, presumably because of the difficulty of preparing pure well-defined substrates in quantity. The availability of highly purified cytidine 2',3'-phosphate (Crook, Mathias & Rabin, 1960*a*) together with a convenient method of assay (Crook, Mathias & Rabin, 1960*b*) prompted a thorough investigation of this problem. A preliminary account of this work has been presented (Herries, 1960).

MATERIALS AND METHODS

A number of buffer compositions not used in this paper but referred to in subsequent papers in this series are included. All materials not listed below were AnalaR chemicals.

Water. Glass-distilled water was passed successively

through columns of De-Acidite FF, Zeo-Karb 225 and 215 (mixed) and Bio-Deminolit (The Permutit Co. Ltd., London). All vessels had previously been thoroughly steamed out. Precautions were taken to protect the final effluent from contamination by atmospheric CO₂.

Sodium chloride and potassium chloride. These were AnalaR or Spectrographically Standardized (Johnson, Matthey and Co. Ltd.). They were heated to approximately 400°, and cooled and stored over P₂O₅.

Hydrochloric acid. AnalaR hydrochloric acid (sp.gr. 1.18) was diluted and distilled according to the method of Vogel (1951*a*, p. 228, method B). Solutions of the required dilution were standardized against 2-amino-2-hydroxy-methylpropane-1,3-diol (tris) (Fossum, Markunas & Riddick, 1951).

Carbonate-free sodium hydroxide solution. A saturated aqueous solution of AnalaR sodium hydroxide was diluted under CO₂-free nitrogen and standardized against standard hydrochloric acid. Potentiometric titration under nitrogen showed that less than 0.3% of carbonate, on an equivalent basis, was present.

Formic acid. AnalaR 90% (v/v) solution was used without further purification.

* Parts 1 and 2: Crook, Mathias & Rabin (1960*a*, *b*).