Reversible Activation of Succinate Dehydrogenase

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1. Treatment of particulate respiratory chain preparations in ways expected to raise or lower the concentration of endogenous soluble low-molecular-weight compounds respectively increased and diminished the capacity of succinate dehydrogenase to become activated reversibly and 'spontaneously' when preparations were diluted in tris acetate buffer and incubated at 37°. 2. Addition of critically low concentrations of recognized activators to preparations that failed to undergo reversible 'spontaneous' activation when incubated at 1 mg. of protein/ml. conferred on them the capacity to do so. 3. Preparations with a diminished tendency to undergo reversible 'spontaneous' activation had an increased tendency to become irreversibly inactivated on prolonged incubation at 1 mg. of protein/ml. in tris acetate. 4. Extraction procedures designed to demonstrate the presence of possible endogenous activators in enzyme preparations failed to reveal a single substance to which such a role could be conclusively attributed. A mixture of compounds was found, however, including certain amino acids that have been shown to act as activators. It is questionable whether these compounds would be present at sufficiently high concentrations to act as activators when enzyme preparations are diluted to 1 mg. of protein/ml. 5. Despite the failure to demonstrate conclusively the presence of endogenous activators, the balance of evidence appears to favour the hypothesis that reversible 'spontaneous' activation of these preparations can best be explained by the presence of such substances, and a scheme describing the mechanism of activation and deactivation of succinate dehydrogenase is discussed in relation to these and other observations.

Activation of succinate dehydrogenase (EC 1.3.99.1) has been observed as a kinetically first-order increase in activity of the enzyme when it is incubated under suitable conditions before assay. The phenomenon was first recognized in soluble preparations of the enzyme and was described in detail by Kearney (1957), who also briefly alluded to a probably similar process in particulate preparations. Kearney found that the activation of the soluble enzyme was dependent on the presence of one of a range of compounds known to be either substrates or competitive inhibitors of the enzyme, but that the attempted removal of the activator (in this case, malonate) by dialysis did not lead to the reverse process of deactivation. The kinetics of activation, and spectral changes of the enzyme, led Kearney to suggest that the process was the result of a conformational change of the enzyme from a form with low catalytic activity to one with high activity, the change taking place as a result of complex-formation between the enzyme and the activator. These observations were summarized by Kearney in terms of the following mechanism:

where E and E* represent respectively the un-activated (low-activity) and activated (high-activity) forms of the enzyme, and A the activator.

Thorn (1962) described an activation of succinate oxidase activity, attributable to activation of succinate dehydrogenase, in particulate preparations. Although this process was similar in many ways to that described by Kearney (1957), it could nevertheless be observed in the absence of added substrates or competitive inhibitors, and in these circumstances slow deactivation was found to take place merely by cooling the enzyme preparation, which had previously been activated by incubation at 37°. Full activity could be regained by re-incubating the deactivated preparation at 37°. Thorn considered that this reversible and apparently spontaneous behaviour was consistent with a temperature-dependent equilibrium between the two forms of the enzyme, and proposed a mechanism summarized in Scheme 1. In Scheme 1 E_U , E_A and E_I represent respectively unactivated, activated and irreversibly inactivated forms of the enzyme, and C the activator. The failure of Kearney to observe activation in the absence of compounds that combined with the active centre of the

Scheme 1.

enzyme was attributed to the relative instability of the soluble enzyme (conversion of E_A to E_I) and the role of activators was thus seen as a protection against irreversible inactivation. The fact that activation could not be reversed in the presence of activators was thought to be due to displacement of the activation-deactivation equilibrium of the enzyme by complex-formation with the activator, which also led to stabilization of the enzyme in its high-activity conformation. It was presumed that Kearney's dialysis procedure was insufficiently effective to remove malonate completely and thus to bring about deactivation.

Kimura, Hauber & Singer (1963) published a short account of the rapid deactivation of succinate dehydrogenase, both in soluble and particulate forms, by gel filtration of activated enzyme through Sephadex G-25. Such deactivated enzyme could be reactivated by a second incubation in the presence of an activator, showing that the loss of activity on gel filtration was reversible and probably due to separation of the enzyme protein from the low-molecular-weight compound present during the first activation period. These investigators thus reasserted the essential role of activators in the activation process, and suggested that the 'spontaneous' activation observed by Thorn (1962) might be due to traces of endogenous activators. They were unable to explain the temperature-dependent reversibility observed by Thorn, however, and suggested that this process might be unconnected with the rapid deactivation observed by themselves on gel filtration.

The present work was undertaken to test these suggestions, and to clarify if possible the relation between the various activation and deactivation phenomena. Since this work was completed, Kimura, Hauber & Singer (1967) have published more extensive data, which complement the findings reported here. During the course of the present work the activating properties of a range of substances, including some hitherto unrecognized as inhibitors of the enzyme, were also investigated. Parts of this work have already appeared in preliminary form (McDonald-Gibson & Thorn, 1967).

MATERIALS AND METHODS

Enzyme preparations. Preparations were of the Keilin & Hartree type as previously described (Thorn, 1962), except

that the enzyme particles were collected by high-speed centrifugation (34 000g for 35 min., cf. Slater, 1957) instead of by acidification to pH 5.5 and low-speed centrifugation. The particles were washed with water at 0°, centrifuged again under the above conditions, and finally suspended at a protein concentration of 20–30 mg./ml. in buffer, pH 7.6 (usually tris acetate, 0.1 M with respect to tris). Where necessary in the text these preparations will be distinguished by the terms 'high-speed' and 'low-speed'. Sometimes the enzyme particles were washed twice, once with water as above and a second time with 0.1 M-tris acetate, before final suspension in the same buffer ('twice-washed' preparations).

Measurements of enzyme activity. Succinate oxidase activity was determined spectrophotometrically at 25° as described previously (Thorn, 1962).

Succinate dehydrogenase activity was measured spectrophotometrically with phenazine methosulphate as primary acceptor and with 2,6-dichlorophenol-indophenol as re-oxidant and terminal acceptor (Redfearn & Dixon, 1961; Arrigoni & Singer, 1962). The assay medium, final vol. 3 ml., contained: tris acetate, pH 7.6, 0.1 M; succinate, 25 mM; KCN, 1 mM; 2,6-dichlorophenol-indophenol, 55 μM; phenazine methosulphate, 2.2 mM (approx. 2 mg./3 ml. of reaction medium); enzyme preparation, approx. 25 μg. of protein. The decrease in extinction at 600 nm., due to reduction of indophenol, was measured against a blank from which substrate, cyanide and dyes were omitted, by using a Unicam SP.800 with a scale expansion of 2 and a chart speed of 1.5 in./min. on an external recorder. The reaction was started by the addition of enzyme, the recording was started 10 sec. later, and the rate was calculated from the change in extinction over the first 1–1.5 min. Enzyme activity was calculated by using an extinction coefficient for 2,6-dichlorophenol-indophenol of 21.6 mm⁻¹ cm.⁻¹ at pH 7.6, (Armstrong, 1964). Although for reliable kinetic data it would be necessary to calculate V_{max} after determining rates at a series of phenazine methosulphate concentrations, it was considered adequate for the present comparative studies to employ a single optimum concentration of the dye. The concentration quoted above gave a rate about 90% of V_{max} , and higher concentrations appeared to be inhibitory.

The protein was determined either as fat-free dry weight (Slater, 1949) or by a biuret method (Cleland & Slater, 1953; Gornall, Bardawill & David, 1949). In parallel determinations consistency between the two methods was demonstrated.

Activation of succinate dehydrogenase. Heart-muscle preparation was diluted to approx. 1 mg. of protein/ml. in one of the following media at 0° and pH 7.6: (a) 0.1 M-tris acetate; (b) 0.1 M-tris acetate containing 37.5 mM-succinate; (c) 0.1 M-phosphate. The first medium was suitable for subsequent examination of 'spontaneous' activation, and the other two were employed for producing maximum activation. Samples of such dilutions were added to assay media for the determination of 'basal' enzyme activity. Activation of the enzyme was then performed by incubation at 37° (for 30 min. with 'spontaneous' activation, and for 10 min. and 20 min. respectively with succinate and phosphate as activators), and a sample was added to the assay medium for the determination of activated enzyme activity. These incubation times were adequate for eliciting the maximum activity in the respective media (cf. Thorn, 1962), although it should be emphasized that the maximum activity

attainable 'spontaneously' was not always as high as that reached with added activators, as is explained in the Results section.

Gel filtration of enzyme preparations. Sephadex G-25 Fine, previously swollen in 0.1M-tris acetate, pH 7.6, was packed in columns and allowed to settle at 4° overnight. Columns were standardized with 0.2% Blue Dextran 2000 (Pharmacia, Uppsala) in 0.9% (w/v) NaCl soln. A portion (1 ml.) of this solution was added to the column and eluted with the same buffer. With a typical column the excluded Blue Dextran appeared after collection of 2.8 ml. of eluate and the bulk of it emerged in the following 3 ml., after which a further 1 ml. could be collected before emergence of NaCl. The time taken for the elution of the high-molecular-weight fraction, and before the low-molecular-weight fraction appeared, was about 15 min. It was assumed that the enzyme particles would behave like Blue Dextran and activators like NaCl. Enzyme preparation was diluted to approx. 10 mg. of protein/ml., activated, and 1 ml. of the cooled suspension was added to the column at 4°. The media and activation times were as described above. Eluted enzyme generally contained 2–3 mg. of protein/ml., with a total recovery of protein of about 60–75%. Before and after column treatment the activation characteristics of the enzyme were determined, i.e. basal activity, activity after 'spontaneous' activation and activity after incubation in the presence of an activator. In all cases a sample of enzyme was transferred as the last addition to the prepared assay medium and the activity was determined immediately.

Extraction of possible endogenous activators from enzyme preparations. Large-scale enzyme preparations were made, starting from 0.75–1.5 kg. of heart-muscle mince. To give the best chance of detection of endogenous materials the preparation procedure was of the 'low-speed' type and terminated at the collection of the loosely packed precipitate of enzyme particles, which was not washed. Samples of the concentrated particles were suspended in tris acetate buffer and shown to undergo 'spontaneous' activation, and it was also found that the residual inorganic phosphate content was too low to account for this.

The initial extraction was based on the method of Bellamy (1962), with ethanolic NH₃ soln. for 35 min. at 60°. In one preparation 12 μ moles of norleucine were added to act as an internal standard for later quantitative amino acid analysis, and in another 48 μ moles of malonic acid were added to test the separation and detection methods for organic acids. After cooling, the mixture was centrifuged (77000g for 30 min.), the supernatant liquid was retained, the residues were resuspended and washed in 50% (v/v) ethanol and, after centrifuging again, the supernatant liquid was combined with the previous one. The combined extracts were concentrated in stages to about 10 ml. at 40° under reduced pressure in a rotary evaporator. The process was interrupted to remove a precipitate of protein by adjusting the pH to about 4.5 with dil. HCl, and centrifuging the flocculent suspension at 100000g for 1 hr. A clear, bright-yellow solution was obtained.

Examination of extracts for the presence of amino acids. The above extract was acidified to pH 2–3 and chromatographed on Zeo-Karb 225 (H⁺ form, bed volume 10 ml. in a 1 cm. diam. column). A fraction containing acidic and neutral compounds was eluted from the column with 50 ml. of 50% (v/v) ethanol and contained the bulk of the yellow compound. It was concentrated by rotary evaporation at

40° under reduced pressure to 10 ml. for later examination. The amino acids were eluted with 20 ml. of aq. 4M-NH₃ soln. After rotary evaporation at 40° under reduced pressure the eluted material was desiccated and weighed. About 50% of the dry weight consisted of amino acids (ninhydrin method of Yemm & Cocking, 1955). Chromatography in one direction on Whatman no. 3 paper in each of two solvent systems (butan-1-ol-acetic acid-water, 12:3:5, and butan-1-ol-pyridine-aq. 3M-NH₃ soln., 4:3:3) showed the presence of a large number of amino acids and a sample of the extract was analysed quantitatively (see the Results section).

Examination of extracts for the presence of organic acids. The concentrate of aqueous ethanol washings from the Zeo-Karb 225 column was adjusted to pH 7–7.6 and applied to a column of Amberlite CG-400 (carbonate form, bed volume 10 ml. in a 1 cm. diam. column). The column was washed with 25 ml. of water to remove neutral substances and the displaced CO₃²⁻ ions and the resin was then removed from the column and treated with 10 ml. of 1.5M-HCl for 15–20 min. The mixture was filtered through a sintered-glass funnel and the resin washed on the funnel with a further 10 ml. of 1.5M-HCl and with five 6 ml. portions of water. The combined filtrate and washings were dried by desiccation over P₂O₅ and NaOH after concentration by rotary evaporation at 40° under reduced pressure, and freeze-drying. The sticky yellow deposit was weighed, and tested as described in the Results section. T.l.c. methods employed for the detection of organic acids are described below.

Silica-gel (Kiesel-gel G containing calcium sulphate binder) plates were prepared, allowed to dry in air for 24 hr. before use, and were not activated by heating. The solvent system was the upper phase of a mixture of 70 ml. of diethyl ether, 10 ml. of formic acid (98–100%, w/w) and 8 ml. of water (cf. Ting & Dugger, 1965). Mixtures of about 10 μ g. each of succinic acid, fumaric acid, malonic acid, malic acid, citric acid, α -oxoglutaric acid and pyruvic acid were well separated. Two methods of detection were employed. In the first, the plate was sprayed with a 0.1% (w/v) solution of 2,6-dichlorophenol-indophenol in 95% ethanol (Passera, Pedrotti & Ferrari, 1964). Pink spots on a blue background were observed, and on keeping the plates in air the keto acid spots tended to turn white. In the second method the plate was sprayed with an acid molybdate reagent (Bandurski & Axelrod, 1951), and exposed to broad-spectrum u.v. light for up to 1 hr. This spray could be applied after the dichlorophenol-indophenol because the pink and blue colours of this reagent faded after a few minutes of treatment. After exposure to u.v. light the hydroxy and keto acids tested gave blue spots on a white background.

RESULTS

Use of succinate oxidase activity as a measure of changes in activity of succinate dehydrogenase. Evidence that changes in succinate oxidase activity observed during activation and deactivation procedures could be attributed to changes in succinate dehydrogenase activity (measured with phenazine methosulphate) have been previously presented (Thorn, 1962). The greater convenience of the succinate oxidase-assay method suggested its routine use as an indicator of changes in succinate

dehydrogenase activity. The validity of this procedure was tested by measuring activation in a number of experiments both by the oxidase assay and by the phenazine methosulphate assay, and the degrees of activation by each method were found to be closely related (Fig. 1). Points in the lower left quadrant indicate that irreversible inactivation took place: this occurred in the absence of added activators after treatment of enzyme in ways expected to lower the concentration of endogenous activators. The equation for the line of least squares was $y = 0.83x + 21$, rather than $y = x$, which would have been expected with exactly corresponding degrees of activation. The departure of the observed line from the expected line is, however, less than the scatter of some individual points. It was concluded that for most purposes the activation observed by the oxidase method gave an adequate index of changes in succinate dehydrogenase activity under the activation conditions employed in this work, but crucial experiments were performed with the succinate-phenazine reductase method.

Characteristics of 'spontaneous' activation in high-speed preparations. In contrast to previous observations (Thorn, 1962) with low-speed preparations, the present series of high-speed preparations showed the phenomenon of 'spontaneous' activation in tris acetate much less clearly. Thus in 17 experiments activation in tris acetate at 37° showed increases in activity averaging only 75% (range 0–163%), compared with maximum increases observed in duplicate samples in phosphate (a recognized activator) averaging 160% (range 21–290%). In no case did activation in tris acetate equal or exceed that in phosphate. Moreover the partially activated enzyme in tris acetate tended to lose activity irreversibly on prolonged incubation (more than 30 min.), and was not then capable of further activation on addition of phosphate and continued

incubation. This type of instability in tris acetate was more marked than had been noted in the previous work, although it was less than the extreme instability observed in borate (Thorn, 1962). These observations underline the fact that a failure to activate is usually accompanied by a tendency towards irreversible inactivation, and show that the behaviour of most of the present preparations was

Fig. 1. Relation of changes in succinate oxidase activity to changes in succinate dehydrogenase activity on subjecting particulate heart-muscle preparations to conditions for activation of succinate dehydrogenase. Oxidase and dehydrogenase activities were determined as described in the Materials and Methods section before and after incubation of preparations at 37° at a concentration of approx. 1 mg. of protein/ml. in 0.1 M-tris acetate, or in 0.1 M-tris acetate containing 37.5 mM-succinate, or in 0.1 M-phosphate (all at pH 7.6), for 30 min., 10 min. and 20 min. respectively. Initial activity was subtracted from final activity, and the difference was expressed as a percentage of initial activity. The line was calculated by the method of least squares.

Table 1. *Activation of succinate dehydrogenase activities in 'high-speed' and 'low-speed' preparations*

A single preparation was carried as far as the first centrifugation step and the supernatant liquid was then divided. Half was submitted to high-speed centrifugation and washing ('high-speed' preparation) and the other half was adjusted to pH 5.5 and submitted to low-speed centrifugation and washing ('low-speed' preparation) (see the Materials and Methods section). Succinate dehydrogenase activities were determined after the treatments indicated. Percentage increases in activity are shown in parentheses, and the bottom line gives a measure of the relative degrees of 'spontaneous' activation in the two preparations.

Treatment	Succinate dehydrogenase activity (μ moles/hr./mg.)	
	High-speed preparation	Low-speed preparation
Kept at 0° (basal activity)	10.1	9.1
Incubation in tris acetate (37°/30 min.)	14.6 (+44.6%)	23.9 (+163%)
Incubation in phosphate (37°/20 min.)	37.3 (+269%)	32.4 (+256%)
% increase in tris acetate $\times$ 100	16.6%	63.7%
% increase in phosphate		

unsuitable for the demonstration of 'spontaneous' reversible temperature-dependent activation, which requires prolonged periods of incubation. As will be shown below, however, the preparations were now suitable for the investigation of conditions under which this type of behaviour could be induced.

The main difference between the present preparations and those used by Thorn (1962) was in the preparative centrifugation method. To test the effect of this change in preparative procedure, the material in one batch was divided, one half then being treated by the 'high-speed' procedure, the other by the 'low-speed' procedure. The activation properties of the two types of preparation were then compared (Table 1). 'Spontaneous' activation of succinate dehydrogenase activity was much more marked in the low-speed preparation than in the high-speed preparation. It is probable that the concentration of endogenous substances in low-speed preparations would be higher than in high-speed preparations, because in the former case the loose flocculation of the enzyme particles at pH 5.5, the fluffy pellet obtained on low-speed centrifugation and the consequent difficulty in removing all the supernatant liquid would all favour the retention of low-molecular-weight compounds that could act as activators. Thus if 'spontaneous' activation in tris acetate were indeed due to the presence of endogenous activators, the above results would find a ready explanation.

Methods expected to alter the concentration of suspected endogenous activators. Gel filtration, first used in this problem by Kimura *et al.* (1963), was applied to a number of preparations. Examples of the activation behaviour, before and after gel filtration, are shown in Table 2. In Expt. A the

limited amount of 'spontaneous' activation of the preparation before Sephadex treatment was abolished after the treatment and the deactivation was too rapid to be accounted for merely by the fall in temperature (cf. Thorn, 1962). In Expt. B the enzyme was applied to the column without prior activation, but the pattern of activation behaviour was similar to that in Expt. A. In both cases an attempt to bring about 'spontaneous' activation of the treated preparation led to a loss of activity, and in other experiments this was shown to be due to irreversible inactivation, i.e. no activity could be restored by further incubation with an activator. Results of gel filtration with eight preparations were consistent with this pattern, although Sephadex treatment did not always lower the activity to the basal level, and maximum activity after Sephadex treatment was not always as high as that observed before.

The effect of repeated washing of enzyme particles is shown in Table 3, in which the activation behaviour of once-washed and twice-washed preparations of the same batch are compared. Repeated washing yielded preparations with a diminished tendency to undergo 'spontaneous' activation and an increased tendency to undergo irreversible inactivation.

Variation of protein concentration during 'spontaneous' activation procedure should correspondingly alter the concentration of suspected endogenous activators, and hence the degree of dissociation of the enzyme-activator complex. Twice-washed preparations, that showed little or no tendency to activate 'spontaneously' when incubated at 1 mg. of protein/ml., were tested for this capacity at higher concentrations (Table 4). The results were consistent with prediction. Behaviour

Table 2. *Activation behaviour of heart-muscle preparations before and after passage through a column of Sephadex G-25*

Expt. A. Heart-muscle preparation was diluted to 12.8 mg. of protein/ml. with tris acetate, pH 7.6, at 0°, and its succinate dehydrogenase activity was determined immediately (basal activity), after 'spontaneous' activation, and after activation by incubation with succinate (see the Materials and Methods section). A portion (1 ml.) of the diluted preparation after 'spontaneous' activation was placed on the column, and 3 ml. of eluate containing 3.5 mg. of enzyme protein/ml. was collected. The activity of this enzyme was determined immediately (basal activity) and after activation either 'spontaneously' or in the presence of succinate. Expt. B. Expt. A was repeated, but in this case the enzyme was applied to the column without prior activation; 3.5 ml. of eluate containing 1.9 mg. of protein/ml. was collected. In both cases the state of the enzyme before passage through Sephadex is indicated by an asterisk.

		Succinate dehydrogenase activity (μ moles/hr./mg.)		
		Basal	After incubation in tris acetate ('spontaneous')	After incubation with succinate
Expt. A.	Before gel filtration	9.7	23.9*	54.7
	After gel filtration	10.3	3.6	48.0
Expt. B.	Before gel filtration	10.4*	15.2	44.5
	After gel filtration	9.8	4.7	44.5

Table 3. *Activation of succinate oxidase activities in once-washed and twice-washed preparations*

A heart-muscle preparation containing 21.9 mg. of protein/ml. was divided. One half was given no further treatment ('once-washed' preparation), and the other half was diluted with 5 vol. of tris acetate, pH 7.6, at 0°, centrifuged at 34000g for 30 min. and the pellet resuspended in tris acetate to give a preparation containing 19.8 mg. of protein/ml. ('twice-washed' preparation). Succinate oxidase activities were determined before activation, and after activation either 'spontaneously' or in the presence of 0.1 M-phosphate. Percentage increases in activity are shown in parentheses.

Treatment	Succinate oxidase activity (μ moles/hr./mg.)	
	Once-washed preparation	Twice-washed preparation
None (unactivated)	8.7	6.8
Incubation in tris acetate (37°/30 min.)	11.9 (+36.8%)	4.9 (-28%)
Incubation in phosphate (37°/20 min.)	17.8 (+105%)	14.8 (+118%)

Table 4. *Effect of degree of dilution of heart-muscle preparation on the ability of succinate dehydrogenase to activate 'spontaneously'*

Twice-washed preparation was diluted in tris acetate to the protein concentrations shown, and incubated for 30 min. at 37°. Succinate oxidase activity was determined before and after this treatment. A control dilution of the enzyme preparation in 0.1 M-phosphate was also incubated for 20 min. at 37°. The protein concentration in the assay was the same (approx. 0.1 mg.) in all cases. Percentage increases in activity are shown in parentheses.

Incubation medium	Concn. of protein (mg./ml.)	Succinate oxidase activity (μ moles/hr./mg.)
(Not incubated)		9.8
Tris acetate	0.92	10.5 (+7.2%)
Tris acetate	1.85	17.4 (+77.6%)
Tris acetate	3.70	20.4 (+108%)
Phosphate	0.92	25.0 (+155%)

of this type with borate buffer as the suspending medium was noted by Thorn (1962), but no explanation was then offered.

These three types of experiment suggest that endogenous activators could be present in our preparations.

Attempts to confer the property of temperature-dependent reversal of 'spontaneous' activation on preparations that did not show this property. The property of the temperature-dependent reversal of 'spontaneous' activation was fully described by Thorn (1962). Owing to the long incubation times

required, and to the instability of the present preparations, the phenomenon could not be demonstrated in this series of experiments. It was reasoned, however, that addition of a very low concentration of a recognized activator might confer the property of temperature-dependent reversal of activation on the present preparations.

The experiment shown in Fig. 2 illustrates that this expectation was fulfilled, a critical concentration of malonate having been added to a preparation that failed to show similar behaviour spontaneously. The form of the experiment can be compared with the spontaneous example illustrated in Fig. 5 of Thorn (1962). The concentration of malonate was critical because with higher concentrations almost no deactivation took place on cooling from 37° to 20° whereas with lower concentrations a substantial degree of irreversible inactivation took place during the lengthy incubation periods. Once more, the connexion between activating and protecting properties is underlined.

Attempts to identify endogenous activators in particulate preparations. The strong circumstantial evidence for the existence in Keilin & Hartree preparations of endogenous activators of succinate dehydrogenase prompted a search for such compounds. During parallel studies (M. B. Thorn & R. G. McDonald-Gibson, unpublished work) many compounds were found to have the property of modifying succinate dehydrogenase activity, and

Fig. 2. Approach of succinate oxidase activity of dilute (approx. 1 mg. of protein/ml.) heart-muscle preparation (which failed to undergo 'spontaneous' activation) to an equilibrium degree of activation at 20° in 0.1 M-tris acetate containing 12 μ M-malonate. O, Course of activation on incubating a sample at 20°; the sample was transferred to 37° at the time indicated by the arrow. Δ , Course of deactivation on incubating a sample, previously warmed at 37° for 10 min., at 20°; the sample was returned to 37° at the time indicated by the arrow.

Table 5. *Quantitative analysis of amino acid content of a large-scale heart-muscle preparation*

Amino acid	Concn. (μ moles/g. of protein of heart-muscle particles from which extract was made)
Aspartic acid	1.39
Threonine	1.60
Serine	1.55
Glutamic acid	2.29
Proline	+
Glycine	1.09
Alanine	3.77
Valine	2.00
Isoleucine	1.69
Leucine	5.97
Tyrosine	1.10
Phenylalanine	2.65
Lysine	2.10
Histidine	0.80
Arginine	1.11
Total	29.11

under suitably chosen conditions could act predominantly as activators, as protectors or as competitive inhibitors. These compounds included certain amino acids, citrate and derivatives of pyrophosphate, all of which are ubiquitous in living tissues and therefore possible candidates for the role of endogenous activators. The amounts of these substances that would survive the various washing procedures involved in preparing the particulate enzyme would nevertheless be very low. Three attempts were made to identify possible endogenous activators, along the lines described in the Materials and Methods section. Extracts were examined for amino acids, organic acids and nucleotide derivatives.

Table 5 shows the results of quantitative analysis of one amino acid fraction, with a single-column Technicon Amino Acid Analyser. By means of an internal standard of norleucine, added at the extraction stage, it was possible to express the results as μ moles of amino acids/g. of heart-muscle-preparation protein. It is thought that physiological significance should not be attributed to these results, owing to the methods employed in making the enzyme preparation and to uncertainty about completeness of extraction and possible losses. The total amino acids detected in this way (29.1 μ moles/g.) compares with an initial quantity of 47.1 μ moles/g., calculated as leucine equivalents, on applying the quantitative ninhydrin method of Yemm & Cocking (1955) to the amino acid extract, but the sum of the results from the Analyser does not include proline and unidentified peaks. The quantity of amino acids found might have contributed towards 'spontaneous' activation when the

preparation was diluted to 1 mg. of protein/ml., but there is no single obvious activator. If histidine had been present at the concentration found for leucine, it could have had an appreciable activating effect (M. B. Thorn & R. G. McDonald-Gibson, unpublished work).

The organic acid fraction was examined by t.l.c. as described in the Materials and Methods section. No acids, other than phosphoric acid which remained near the origin, were detected in this fraction, although malonic acid added at the extraction stage was easily detected on the chromatogram. The amount of phosphate present in the original enzyme had been shown to be insufficient to account for 'spontaneous' activation. On the other hand, the amount of malonate added was enough to have produced an activating effect on dilution of the enzyme preparation to 1 mg. of protein/ml. Thus had the preparation contained an endogenous anion that was as effective an activator as malonate, and had this anion been responsible for 'spontaneous' activation, the procedure described should have detected its presence.

The acid fractions contained the bulk of the yellow colour associated with the original aqueous-ethanol extract of heart-muscle preparation. Absorption peaks at 267, 373 and 445 nm., and tests for pentose, and for inorganic and combined phosphate, suggested the presence of FMN. Examination of thin-layer chromatograms under u.v. light showed a number of fluorescent spots that were considered to be breakdown products of FMN. Preliminary experiments also indicated the possible presence of other nucleotides. It is not possible as yet to attach any definite significance, either physiological or in connexion with 'spontaneous' activation, to these observations. A role for nucleotides as activators cannot, however, be ruled out (M. B. Thorn & R. G. McDonald-Gibson, unpublished work).

These extraction experiments thus failed to show conclusive evidence for the presence of an endogenous activator, although a mixture of compounds, notably amino acids, was present.

DISCUSSION

The evidence reported above strongly suggests that the 'spontaneous' activation previously observed by Thorn (1962) was due to the presence of endogenous activators in heart-muscle preparations. Much of this work confirms the observations of Kimura *et al.* (1963, 1967), but the findings that 'spontaneous' activation is reversible by gel filtration, and that reversible activation, leading to a temperature-dependent equilibrium, can be induced in a preparation failing to show this property merely by adding a critically low concentration of a

recognized activator (competitive inhibitor), are significant. As yet no endogenous activator has been identified in heart-muscle preparations, but the isolation and detection methods could have failed to reveal volatile acids or unstable compounds. Kimura *et al.* (1963, 1967) postulated the presence in heart-muscle particles of a very firmly bound endogenous activator, which could not be entirely removed by gel filtration unless it had been 'displaced' by prior treatment of the enzyme with succinate. It may be that oxaloacetate could fulfil such a role since it combines strongly with the enzyme (K_i in the range 1–10 μM), but it may not act as a typical activator because the inhibition is probably not of a simple competitive nature (cf. Zeylemaker & Slater, 1967), and the amount in tissues is very small.

Kimura *et al.* (1967) refer to a 'thermal activation', which represented a 'modest degree' of activation (i.e. a final activity apparently of up to one-third of that attainable in the presence of succinate as activator) and which was complete in 2 min. at 38°. They equated this phenomenon with the 'spontaneous' activation observed by Thorn (1962), which, however, resulted in an activity up to 90% of maximum, and took up to 30 min. [cf. Fig. 3 of Thorn (1962)]. Kimura *et al.* (1967) were therefore probably not observing the same form of 'spontaneous' activation, and according to our present views there is indeed no such process. The explanation of their observations may be that they employed a supposedly inert buffer (67 mM-imidazole) as an incubation and assay medium. However, McDonald-Gibson (1967) has found that imidazole is a weak inhibitor of succinate dehydrogenase (although this is scarcely evident at the optimum substrate concentrations employed in routine assays), has a small protective action against inhibition of the enzyme by *p*-aminophenyl-arsenoxide and a definite capacity to act as an activator (between 45 and 75% of maximum activities were attained in the presence of imidazole, 10 mM and 100 mM, on incubation at 37° for 15 min.). If these workers had employed tris buffer as an incubation and assay medium it is probable that they would have observed no 'thermal activation', and that irreversible inactivation might have taken place.

The mechanism of activation (and deactivation) of succinate dehydrogenase shown in Scheme 2 is now proposed in place of those offered by Kearney (1957), Thorn (1962) and by Kimura *et al.* (1967). It represents a combination of the schemes of Kimura *et al.* (reactions I and II) and of Thorn (reactions III, IV and V), with certain restrictions discussed below. The cycle of reactions (I), (II), (III) and (IV) is shown as fully reversible; reactions (I) and (III) are probably in rapid equilibrium, but

reactions (II) and (IV) are considered to be slow, and indeed negligible in the directions represented by broken arrows. Thus enzyme in the unactivated state (E_U) on the left-hand side of the Scheme becomes activated predominantly by reaction (II) (forward), i.e. when in combination with an activator (C, a compound acting as a substrate or competitive inhibitor). Conversely, enzyme in the activated state (E_A) on the right-hand side of the Scheme becomes deactivated primarily by reaction (IV) (forward). In other words, the Scheme expresses the idea that the most favoured conformation of the free enzyme is the unactivated form E_U , whereas the preferred conformation of the enzyme-activator complex is the activated form $E_A C$. Reactions (V) and (VI) are included to take into account the fact that irreversible inactivation (formation of E_I) is often observed when activators are absent, or are present only in suboptimum concentrations; it is not possible at present to assess the relative contributions of reactions (V) and (VI) to irreversible inactivation.

The considerable effect of temperature on activation is likely to be due to its effect on reaction (II) (forward). To account for the establishment of a temperature-dependent equilibrium between activated and unactivated forms of the enzyme it is necessary only to postulate that the temperature coefficient of reaction (II) (forward) is greater than that of reaction (IV) (forward) in the circumstance that the concentration of an activator is low, i.e. substantial amounts of enzyme exist both in free and combined forms. This condition was fulfilled in the experiment documented in Fig. 2, since if 7 μM is accepted as the K_i of malonate (Thorn, 1953) it can be calculated that the ratio of free to combined enzyme was about 1:2. A basis for discussion of this activation-deactivation mechanism, derived from equilibrium kinetics, is presented in the Appendix.

The suggested Scheme, unlike that of Kimura *et al.* (1969), can account for the 'irreversibility' of activation of succinate dehydrogenase in the presence of optimum concentrations of activators (Kearney, 1957; Thorn, 1962), the failure of the enzyme to reach full activation in the presence of

Scheme 2.

suboptimum concentrations of activators (Kearney, 1957), the reversal of activation by gel filtration and by other treatments designed to wash out or to dilute existing endogenous activators (Kimura *et al.* 1963, 1967; and the present work), the establishment of a temperature-dependent equilibrium in the presence of low concentrations of added activators (the present work) or with endogenous activators (Thorn, 1962, as interpreted in the present work), and the tendency of the enzyme to undergo irreversible inactivation when it is in the presence of only low concentrations of activators, either added or endogenous.

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REFERENCES

Armstrong, J. McD. (1964). *Biochim. biophys. Acta*, **36**, 194.
Arrigoni, O. & Singer, T. P. (1962). *Nature, Lond.*, **193**, 1256.

Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.
Bellamy, D. (1962). *Biochem. J.* **82**, 218.
Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Kearney, E. B. (1957). *J. biol. Chem.* **229**, 363.
Kimura, T., Hauber, J. & Singer, T. P. (1963). *Biochem. biophys. Res. Commun.* **11**, 83.
Kimura, T., Hauber, J. & Singer, T. P. (1967). *J. biol. Chem.* **242**, 4987.
McDonald-Gibson, R. G. (1967). Ph.D. Thesis: University of London.
McDonald-Gibson, R. G. & Thorn, M. B. (1967). *Biochem. J.* **104**, 35P.
Passera, C., Pedrotti, A. & Ferrari, G. (1964). *J. Chromatog.* **14**, 289.
Redfearn, E. R. & Dixon, J. M. (1961). *Biochem. J.* **81**, 19P.
Slater, E. C. (1949). *Biochem. J.* **45**, 1.
Slater, E. C. (1957). *Symp. Soc. exp. Biol.* **10**, 110.
Thorn, M. B. (1953). *Biochem. J.* **54**, 540.
Thorn, M. B. (1962). *Biochem. J.* **85**, 116.
Ting, I. P. & Dugger, W. M., jun. (1965). *Analyt. Biochem.* **12**, 571.
Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.
Zeylemaker, W. P. & Slater, E. C. (1967). *Biochim. biophys. Acta*, **132**, 210.

APPENDIX

On the basis of the cyclic activation-deactivation scheme given in the Discussion section of the above paper it is possible to derive an expression for the fraction of activated enzyme present ($[E_A] + [E_A C]$ as a fraction of $[E_A] + [E_A C] + [E_U] + [E_U C]$) in terms of the activator concentration and of the various equilibrium and velocity constants. The reactions of association and dissociation of activator (reactions I and III) can be taken as being very rapid compared with the reactions of activation (reaction II, forward) and of deactivation (reaction IV, forward). For example, at 38°, k_{+1} and k_{-1} for succinate are of the order respectively of $10^4 \text{ l. mole}^{-1} \text{ sec.}^{-1}$ and 1 sec.^{-1} (cf. Slater & Bonner, 1952; Thorn, 1953), whereas from data given by Thorn (1962) it can be estimated that, at 38°, k_2 (velocity constant for activation by reaction II, forward) would be about 0.006 sec.^{-1} . In addition, reactions (II) (backward) and (IV) (backward) are assumed to be insignificant, and the complications of irreversible inactivation (reactions V and VI) are neglected. It can then be shown that:

$$\text{Fraction of activated enzyme} = \frac{k_2}{k_2 + k_4 \cdot \frac{K_c}{[C]}} \quad (1)$$

where k_2 and k_4 are the velocity constants of reactions (II) (forward) and (IV) (forward) re-

spectively, $[C]$ is the concentration of activator, and K_c is the enzyme-activator dissociation constant (it is assumed that this has the same value for both activated and unactivated forms of the enzyme). This equation predicts, in accord with experimental observations, that (i) when $[C]$ is very large compared with K_c , the fraction of activated enzyme approaches 1, (ii) when $[C]$ is vanishingly small the fraction of activated enzyme approaches zero, and (iii) when $[C]$ is of the same order of magnitude as K_c , the fraction of activated enzyme depends largely upon the relative magnitudes of k_2 and k_4 , and if k_2 increases with temperature more rapidly than does k_4 then the fraction of activated enzyme will rise with increase in temperature.

For a linear type of activation scheme such as the following:

it can similarly be shown that:

$$\begin{aligned} \text{Fraction of activated enzyme} \\ = \frac{k_{+2}}{k_{+2} + k_{-2} \left(\frac{K_c^U + [C]}{K_c^A + [C]} \right)} \end{aligned} \quad (2)$$

where k_{+2} and k_{-2} are respectively the velocity constants for the forward and reverse of reaction (II), and K_c^U and K_c^A are the enzyme-activator dissociation constants for reactions (I) and (III) respectively. From this equation it can be predicted that (i) when $[C]$ is very large compared with both dissociation constants, k_{+2} must be at least 10 times k_{-2} in order that 90% or more of the enzyme can be converted into the activated form, (ii) with these relative values of k_{+2} and k_{-2} , and as $[C]$ falls to very low values, K_c^U would then have to be of the order of 100 times K_c^A in order that a limiting amount of only 10% of enzyme remained in the activated form, and (iii) when $[C]$ is in the range of values of K_c^U and K_c^A , variation of the fraction of activated enzyme with temperature would depend largely on considerable changes in the relative values of k_{+2} and k_{-2} with temperature (perhaps modified by temperature-dependent changes in K_c^U and K_c^A).

Although equations (1) and (2) do not enable a conclusive choice between the cyclic and linear mechanisms to be made, certain properties of these mechanisms are emphasized. For example, it is

clear that the linear mechanism depends crucially on there being a large difference between K_c^U and K_c^A . In view of the fact that the K_m values for the unactivated and activated enzymes are of similar magnitudes, it would not be expected that there are such large differences in the enzyme-activator dissociation constants. Indeed, K_i values for inorganic phosphate, measured with the same enzyme preparation before and after activation, were of the same order of magnitude (R. G. McDonald-Gibson, unpublished work). Small differences in enzyme-activator dissociation constants do not alter the validity of the general conclusions about the cyclic mechanism. Hence we conclude that the cyclic scheme is more satisfactory in accommodating the present range of experimental observations.

REFERENCES

- Slater, E. C. & Bonner, W. D., jun. (1952). *Biochem. J.* **52**, 185.
Thorn, M. B. (1953). *Biochem. J.* **54**, 540.
Thorn, M. B. (1962). *Biochem. J.* **85**, 116.