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Control of Succinate Dehydrogenase in Mitochondria*

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ABSTRACT: In intact, respiring mitochondria succinate dehydrogenase activity undergoes rapid and extensive changes in response to the metabolic state. Highest succinate dehydrogenase activity is observed in state 4, when coenzyme Q (CoQ) is largely in the reduced state. On transition to state 3 rapid deactivation of the enzyme occurs, in line with data in the literature showing oxidation of CoQ_{10} under these conditions. Lowest activity is observed in state 2 or in the presence of uncouplers, which cause almost complete oxidation of reduced CoQ_{10}. These findings indicate that the activation of the dehydrogenase by reduced CoQ_{10} in membranes also operates in intact mitochondria and is one of the factors governing succinate dehydrogenase activity. The data also help explain reports in the literature of succinate accumulation in state 3 and in the presence of uncouplers and its metabolic removal in state 4. In addition to activation by reduced CoQ_{10} in mitochondria, the enzyme is also activated by succinate and by ATP or a compound in equilibrium with ATP. ATP-induced activation does not seem to involve oxalacetate removal and is not mediated by the energy conservation system, since it is not oligomycin sensitive. In submitochondrial particles and in complex II neither ATP nor GTP seems to activate the enzyme but ITP and IDP do. Activation of succinate dehydrogenase by succinate, substances leading to CoQ_{10} reduction, or ATP occurs more rapidly and with a lower activation energy in mitochondria than in submitochondrial particles or soluble preparations. These observations indicate that the dehydrogenase is under efficient multiple control in intact mitochondria.

It is well established that in soluble or membranal preparations succinate dehydrogenase is converted by substrates and substrate analogs from an unactivated (deactivated) to an activated form (Kearney, 1957) and that it returns to the deactivated state on removal of the activator (Kimura *et al.*, 1967). The possibility that this reversible activation may be of regulatory significance in cell metabolism has been pointed out (Singer, 1968) and became very likely when it was discovered (Gutman *et al.*, 1971a,b) that reduced CoQ_{10}[1] and substances which lead to the reduction of CoQ_{10} in membrane preparations rapidly activate the enzyme, providing thereby a physiologically occurring activator whose concentration changes rapidly and extensively in metabolic transitions (Kröger and Klingenberg, 1966).

It was of interest to examine the extent to which agents

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[1] Abbreviations used are: CoQ_{10}, coenzyme Q_{10}; ETP and ETP_H, nonphosphorylating and phosphorylating preparations of the inner membrane; PMS, phenazine methosulfate; STM buffer, sucrose–Tris–Mg buffer.

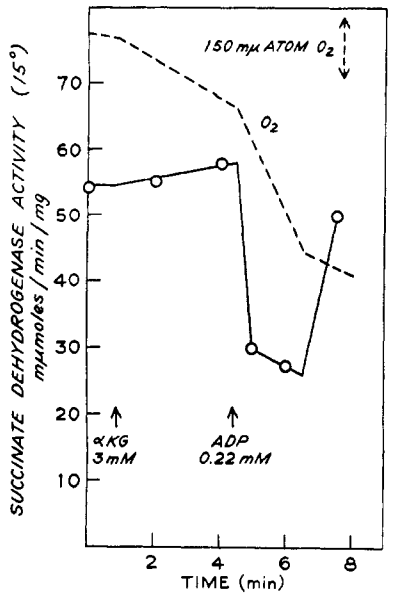

FIGURE 1: Variation of succinate dehydrogenase activity in rat liver mitochondria in states 3 and 4. Conditions: 2.55 mg of protein in 1.5 ml of 230 mM mannitol–70 mM sucrose–20 mM Tris–5 mM KP_i–5 μM EDTA, pH 7.4, at 30°. The dashed line indicates respiration on 3 mM α-ketoglutarate at 30°, the solid line the succinate dehydrogenase activity at 15° of aliquots removed at intervals and assayed immediately.

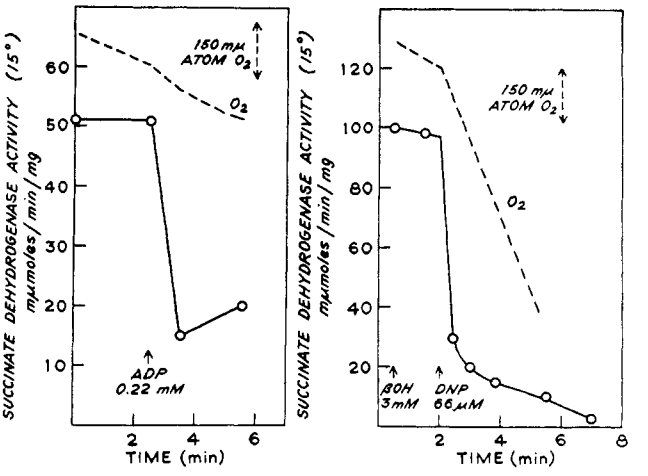

FIGURE 2: Left side, deactivation of succinate dehydrogenase of rat liver mitochondria respiring on endogenous substrate. Conditions were as in Figure 1, except that the protein concentration was 3.5 mg/ml and no substrate was added. Right side, deactivation of succinate dehydrogenase of rat liver mitochondria respiring on β-hydroxybutyrate (β-OH) by 2,4-dinitrophenol (DNP). Conditions were as in Figure 1, except that the protein concentration was 2.5 mg/ml.

that are known to cause extensive fluctuations in the redox state of CoQ_{10} (and, hence, in the prevailing concentration of $CoQ_{10}H_2$) affect succinate dehydrogenase activity in tightly coupled mitochondria. It was found (Gutman *et al.*, 1971c) that in liver mitochondria respiring on a variety of substrates the state 4 → 3 transition is accompanied by rapid and extensive deactivation of succinate dehydrogenase and that the enzyme becomes activated again as ATP accumulates. Deactivation was even more extensive in state 2 and was almost complete when uncouplers were added. All this is in accord with the expected fluctuations in the CoQ_{10}:$CoQ_{10}H_2$ ratio in these metabolic transitions. Moreover evidence was obtained that in mitochondria not only succinate and reduced CoQ_{10} but also ATP (or a compound in equilibrium with it) causes rapid activation of the dehydrogenase. The effect cannot be explained on the basis of oxalacetate removal and is not mediated by the oxidative phosphorylation system, since it is oligomycin insensitive.

The present paper is a detailed account of this work and also presents evidence that in preparations of the inner mitochondrial membrane and of complex II, ITP and IDP but not ATP or GTP activate the enzyme.

Materials and Methods

Liver mitochondria were prepared by the method of Schnaitman and Greenawalt (1968) from Sprague–Dawley rats, which had been fasted for 24 hr. Oxygen uptake was monitored with a Clark electrode at 30° in the medium specified by these authors. Rat heart mitochondria were isolated according to Pande and Blanchaer (1971) and were suspended in their medium for oxygen-uptake measurements and activation studies. The respiratory control on pyruvate plus malate averaged 8. Unless otherwise stated activation was performed either in the Oxygraph chamber thermostated at 30°, using the assay media specified by the authors above, or in test tubes equipped with a magnetic stirrer and maintained at 30°. The usual protein concentration during activation was 1 mg/ml. Samples of 100 μl were removed and pipetted into cuvets containing the reaction mixture for succinate dehydrogenase, which were maintained at 15°. The assay mixture contained 60 mM KP_i (pH 7.6), 1 mM KCN, 52 μM 2,6-dichlorophenolindophenol, and 0.1 mM PMS. $CaCl_2$ (0.75 mM) and purified phospholipase A (10 μg, 600 units/mg) were included to permit free penetration of PMS. Lysis of the mitochondria was complete within a few seconds and assay traces were linear. Succinate dehydrogenase assays were according to Arrigoni and Singer (1962), with succinate used to start the reaction, except that the fixed PMS concentration specified above was used.[2] Each batch of mitochondria was checked for full succinate dehydrogenase activity by activating the complete reaction mixture, including 20 mM succinate but no electron acceptors, for 7 min at 38° prior to cooling the cuvets to 15° and starting the reaction by the addition of dyes. The resulting activity is specified in certain figures as activation by succinate.

ATP, ADP, ITP, and IDP were products of Pabst Laboratories, cyclic 3′,5′-AMP and cyclic 3′,5′-IMP of Sigma Chemical Co. The purity of ITP was checked by thin-layer chromatography in ethanol–1 M ammonium acetate, pH 3.8 (7:3, v/v). Other methods were as in the previous paper in this series (Gutman *et al.*, 1971b).

Results

Deactivation of Succinate Dehydrogenase Initiated by ADP and Uncouplers. In rat liver mitochondria, as isolated, the dehydrogenase is almost entirely in the activated form. Under conditions known to cause extensive oxidation of $CoQ_{10}H_2$, rapid deactivation of the enzyme occurs. Figure 1 shows the

[2] Cysteinesulfinate was included in some but not all activity determinations, since it did not affect the measured activity, probably because initial rates were measured and because in lysed mitochondria the NAD^+ concentration is too low to permit oxalacetate formation.

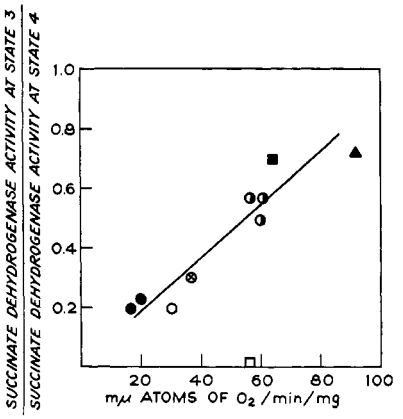

FIGURE 3: Relation of the respiration of rat liver mitochondria on various substrates to the deactivation of succinate dehydrogenase in the state 4 → 3 transition. Conditions: rat liver mitochondria (3.5 mg/ml) were suspended in the medium of Figure 1 in the presence of various substrates (all 3 mM) without added ADP. Aliquots were removed for assay of succinate dehydrogenase. After transition to state 3 initiated with 0.3 mM ADP, respiration was recorded and samples were again removed for determination of succinate dehydrogenase activity. The abscissa denotes respiration in state 3, the ordinate the ratio of succinate dehydrogenase activity in state 3:state 4. (●) Endogenous substrate, (○) choline, (⊗) α-glycerophosphate, (◑) α-ketoglutarate, (▲) succinate, (■) β-OH-butyrate, and (□) β-OH-butyrate plus DNP.

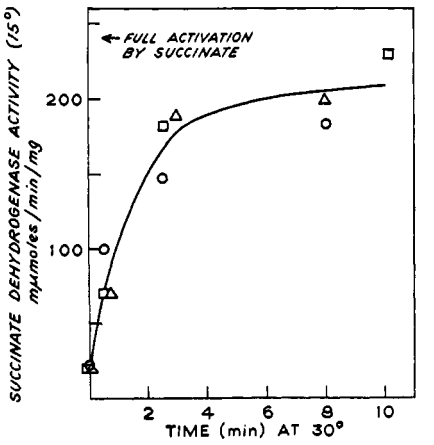

FIGURE 4: Activation of succinate dehydrogenase in rat heart mitochondria initiated by ATP. The mitochondria were suspended in the buffer of Pande and Blanchaer (1971) at 1 mg of protein/ml at 30° and 1 mM ATP was added at 0 time. Aliquots were assayed for succinate dehydrogenase activity as in Figure 1. Without ATP no change in succinate dehydrogenase activity occurred. (△) ATP alone; (□) ATP + oligomycin (2 μg/mg of protein); (○) ATP + piericidin A (1.25 mμmole/mg of protein).

progress of deactivation in the state 4 → 3 transition, initiated by ADP. The decline in succinate dehydrogenase activity is only partial and does not reach a low enough level that it could become rate limiting in the Krebs cycle. The return of activity as ADP is depleted is caused in part by the reduction of CoQ_{10} in return to state 4 (Klingenberg, 1968), in part by the activating effect of the ATP which has been formed, as discussed later in this paper.

Deactivation is more extensive if ADP is added in the absence of exogenous substrate (Figure 2, left side) and even more extensive in the presence of 2,4-dinitrophenol (Figure 2, right side). The decline in dehydrogenase activity in these experiments is not an inactivation, since brief incubation with succinate in the presence of cyanide caused a rapid return of the original activity. Figure 3 summarizes the results of many experiments on the state 4 → 3 transition in rat liver mitochondria respiring on various substrates, denoted by different symbols. There is an inverse relation between the rate of respiration in state 3 and the extent of deactivation of succinate dehydrogenase. The observations in Figures 1–3 are in accord with data in the literature showing that ADP causes severalfold increases in the CoQ_{10}:$CoQ_{10}H_2$ ratio and that uncouplers cause almost complete oxidation of endogenous $CoQ_{10}H_2$ (Kröger and Klingenberg, 1966; Klingenberg, 1968), so that deactivation of succinate dehydrogenase would be expected as a result of the removal of the agent which stabilizes it in the activated conformation (Kimura *et al.*, 1967; Gutman *et al.*, 1971b).

Activation by ATP. The observation that the deactivation in state 3 levels off well before ADP is exhausted and, hence, before the reduction of CoQ_{10} characteristic of state 4 is expected to begin (Figure 1) suggested that ATP may act as another activator of the enzyme. Activation of succinate oxidation in yeast mitochondria has, in fact, been reported by Gregolin and Scalella (1965) who viewed it as a direct effect of ATP, but others regarded the effect as being due to the removal of inhibitory oxalacetate (see review by Greville, 1966, and Papa *et al.*, 1968). In order to avoid such uncertainties it was essential, therefore, to examine the effect of ATP under conditions which preclude accumulation of oxalacetate and to ensure that any activation initiated by ATP is not due to CoQ_{10} reduction, since ATP is known to increase the redox state of the quinone in certain metabolic states.

Activation by ATP was studied in rat heart mitochondria, since in these mitochondria, as isolated, succinate dehydrogenase is extensively deactivated without additional treatment. The experiments of Figure 4 were conducted in the absence of exogenous substrate. It is seen that the addition of 1 mM ATP caused activation of succinate dehydrogenase and that the inclusion of piericidin A to inhibit oxidation of endogenous substrate and consequent reduction of CoQ_{10} did not affect the activation. The effect of ATP does not seem to involve the energy conservation system, since oligomycin did not interfere with the activation. The lack of oligomycin sensitivity also precludes the interpretation that ATP acted by causing reduction of endogenous CoQ_{10}.

Although the rate of activation initiated by ATP in the experiments of Figure 4 is not fast enough for the effect to be interpreted as removal of endogenous oxalacetate but is compatible with activation of the dehydrogenase in mitochondria by other agents at this temperature, the experiments of Figure 5 were performed to rule out the possibility that ATP merely reverses oxalacetate inhibition. It may be seen that in the presence of glutamate[3] to transaminate with any oxalacetate present activation occurred at as low as 6 μM ATP concentration. Arsenite and piericidin A were included to prevent the oxidation of any α-ketoglutarate which may have been formed, which could lead to the reduction of NAD^+ and, hence, of CoQ_{10} and to the formation of succinate.

It should be emphasized that these experiments do not prove that ATP itself is the activator, since the possibility cannot be ruled out that ATP is in equilibrium in the mitochondria with another substance which is the direct activator.

[3] Since respiration on malate was enhanced more by glutamate than by cysteinesulfinate, the former was used to prevent oxalacetate accumulation in experiments of this type.

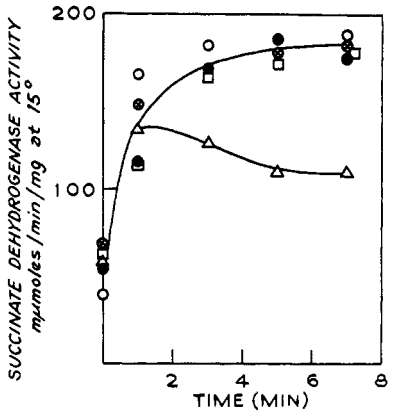

FIGURE 5: Activation of succinate dehydrogenase at varying ATP concentrations. Rat heart mitochondria, suspended as in the experiment of Figure 4, were incubated for 1 min at 30° with 1.2 mμmoles of piericidin A/mg, 1 mM arsenite, and 2.5 μg of oligomycin A/mg; then 10 mM glutamate was added and activation was initiated by adding ATP. The ATP concentrations were (○) 3.15 mM, (●) 0.315 mM, (⊗) 0.189 mM, (□) 0.019 mM, and (Δ) 0.006 mM. In the absence of ATP no change in activity was observed.

Comparison of Activations Initiated by ATP and by NAD⁺-Linked Substrates. Although the experiments of Figures 1–3 strongly suggest that reduced CoQ_{10} is an activator of succinate dehydrogenase in intact mitochondria, just as it is in membrane preparations, it was desirable to obtain additional evidence for this. In the experiments of Figure 6 rat heart mitochondria were used, since these permitted study of the process in the forward direction without prior deactivation. Pyruvate plus malate were used to reduce endogenous CoQ_{10} and cyanide was included to prevent cycling and consequent accumulation of ATP by oxidative phosphorylation. Normal activation, to the same level as observed with succinate as activator, is evident. Again, the inclusion of glutamate or of cysteinesulfinate in the assay to transaminate with any oxalacetate present did not alter the results.

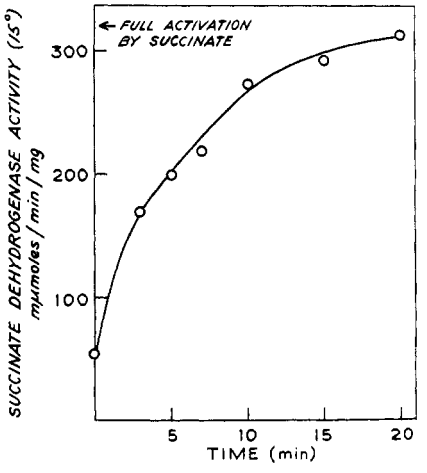

FIGURE 6: Activation of succinate dehydrogenase in rat heart mitochondria initiated by pyruvate + malate in the presence of KCN. The mitochondria were suspended in the medium of Pande and Blanchaer (1971) at 0.48 mg of protein/ml; 1 mM KCN was added, and activation was initiated by 3 mM each of pyruvate and malate at 15°. At intervals aliquots were removed and immediately assayed for succinate dehydrogenase activity (shown on ordinate). The O_2 uptake of the preparation on pyruvate + malate at 30° was 500 mμatoms of O_2/min per mg and the respiratory control ratio 6.

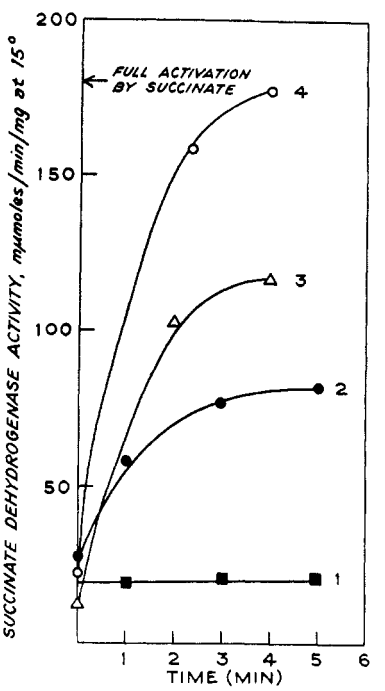

FIGURE 7: Comparison of activation of succinate dehydrogenase of rat heart mitochondria by NAD⁺-linked substrates and ATP. Rat heart mitochondria, suspended as in Figure 4, were treated for 1 min with 20 μM dinitrophenol and 0.3 mM arsenite. Then 5 μg of oligomycin/mg of protein was added and incubation at 30° was continued for 5 min. Zero time indicates the sample taken immediately *before* the addition of substrate or ATP. Curve 1, activation in the presence of 5 mM malate plus 10 mM glutamate; curve 2, activation with 5 mM malate, 10 mM glutamate, plus 3 mM ATP; curve 3, activation in 5 mM malate plus 10 mM glutamate plus 1 mM KCN; curve 4, 5 mM malate, 10 mM glutamate, 1 mM KCN, plus 3 mM ATP.

An approach to estimating the relative efficiency of activation by $CoQ_{10}H_2$ and ATP, respectively, is presented in Figure 7. By virtue of the fact that in this particular experiment neither activation initiated by ATP nor by NAD⁺-linked substrates went to completion, the extent of inactivation by the two types of modulators of the enzyme could be compared. Rat heart mitochondria were incubated with dinitrophenol to deplete endogenous substrate and ATP as much as possible, then oligomycin was added to block the ATPase. Under these conditions malate plus glutamate, in the presence of arsenite, gave no activation (curve 1). This is as expected, since the conditions were aerobic and no inhibitor of the respiratory chain was present, so that significant reduction of endogenous CoQ_{10} would not be expected. On the other hand, when cyanide was included to block cytochrome oxidase, extensive activation was observed (curve 3). The addition of ATP (along with substrate) also gave significant activation (curve 2). The presence of substrate, ATP, and of cyanide gave maximal activation (curve 4); in fact, curve 4 is essentially a summation of curves 2 and 3. This differential activation by ATP and reduced CoQ_{10} was not always observed, since in many preparations either agent alone produced maximal activation.

Although the insensitivity of the ATP-triggered activation to oligomycin would by itself indicate that ATP does not activate by reducing CoQ_{10}, a more direct experiment was designed to test this point. If ATP activated by altering the ratio CoQ_{10}:$CoQ_{10}H_2$, the inclusion of even a relatively low concentration of ADP along with ATP would minimize or

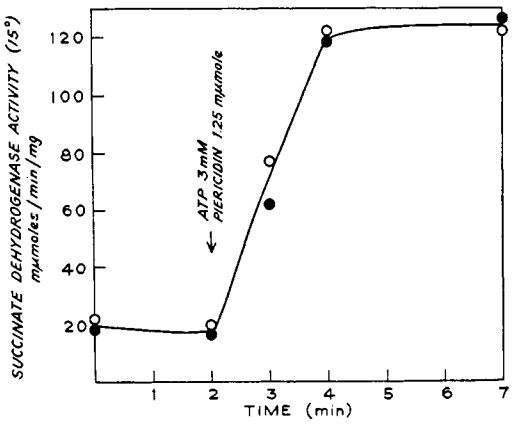

FIGURE 8: Activation by ATP in the presence and absence of added ADP. Rat heart mitochondria, suspended as in Figure 4, were preincubated for 2 min at 30° with 1.25 mμmoles of piericidin/mg of protein and 3 mM ATP was added at the point indicated by the arrow. Aliquots were taken at intervals for determination of succinate dehydrogenase activity. Solid circles, no ADP; open circles, 0.34 mM added ADP.

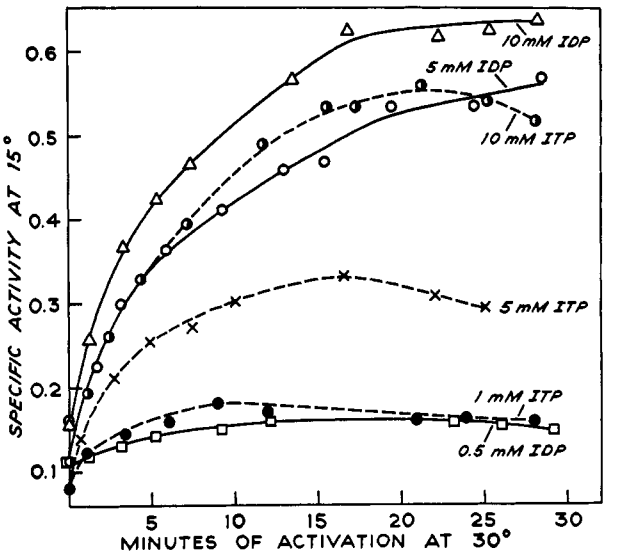

FIGURE 9: Activation of succinate dehydrogenase in ETP_H by ITP and IDP. ETP_H, deactivated by washing with and suspended in 0.18 M sucrose–50 mM Tris–5 mM $MgSO_4$ buffer (pH 7.4), was incubated at 30° with the indicated concentrations of nucleotides. Samples were removed and immediately assayed for succinate dehydrogenase activity at 15°. Note that no correction was made for the possible inhibitory effects of ITP and IDP in succinate–PMS assays. Maximal activation reached was close to that obtained with succinate as activator.

abolish activation by the latter. It is known that state-3 conditions prevail until the phosphate potential, [ATP]/[ADP][P_i] rises to about 10^4 (Chance and Williams, 1956; Chance and Hollunger, 1961), *i.e.*, CoQ_{10} would remain largely in the oxidized state until this value is exceeded. In the experiment illustrated in Figure 8 no substrate was added and the oxidation of endogenous substrate was blocked with piericidin. Activation by 3 mM ATP alone and 3 mM ATP plus 0.34 mM ADP were found to be identical. Under these conditions the phosphate potential is about 1.7×10^3. Since no substrate was present, state 2 conditions prevailed and, hence, the effect of ATP could not involve changes in the redox state of the CoQ_{10} pool.

Activation by Nucleotides in Membrane Preparations. In submitochondrial particles (ETP or ETP_H) ATP and GTP cause little or no activation of the dehydrogenase. On the other hand, activation by ITP and IDP, reaching nearly the same level as activation by succinate, was noted in ETP_H (Figure 9). IDP appears to be more effective than ITP; although not shown in the figure 5′-AMP, 5′-IMP, cyclic 3′,5′-IMP, and cyclic 3′,5′-AMP caused no significant activation at 5–10 mM concentrations.

Although the samples of ITP used in these experiments contained only traces of IDP as judged by thin-layer chromatography, and a sample of ITP chromatographed on Dowex 1-formate to remove IDP and other conceivable impurities proved to be effective as an activator, the possibility could not be ruled out that enzymatic hydrolysis of ITP to IDP occurs in the membrane preparation and that, therefore, IDP is the actual activator. For this reason the effect of ITP was also tested in a preparation ofcomplex II, which is a highly purified particulate form of succinate dehydrogenase (Ziegler and Reiske, 1967) and, as such, may be expected to be relatively free from ATPase. Figure 10 shows that the succinate dehydrogenase in this particle, 17% activated at the start, was fully activated by ITP. (The slow decline in the activity of the succinate-activated control was due to inactivation at 30°.) Preliminary results with a highly purified soluble preparation of the dehydrogenase (Singer *et al.*, 1956) indicate that the enzyme may be also activated by IDP and ITP in this form.

Effect of Activation of Succinoxidase Activity of Intact Mitochondria. In the foregoing experiments modulation of the activity of succinate dehydrogenase was monitored by the PMS assay. Although this is probably the most valid measure of the state of activation of the enzyme, it was of interest to ascertain the extent to which the cycle of activation–deactivation influences succinoxidase activity in tightly coupled mitochondria. Figure 11 compares succinoxidase activity under three different conditions of activation. In the curve denoted by shaded circles rat heart mitochondria were

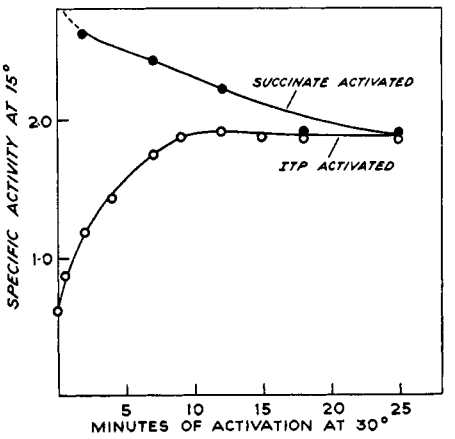

FIGURE 10: Activation of succinate dehydrogenase in complex II by ITP. The complex II preparation was 83% deactivated by passage through Sephadex G-25 in the absence of succinate, suspended in the same buffer as in Figure 9 at 0.2-mg/ml concentration and warmed for 5 min at 30° to ensure deactivation prior to the addition of 10 mM ITP where shown. The upper curve shows the decay of a succinate activated sample at 30° (no dithiothreitol present). Note that no correction was made for possible interference of ITP in succinate–PMS assays.

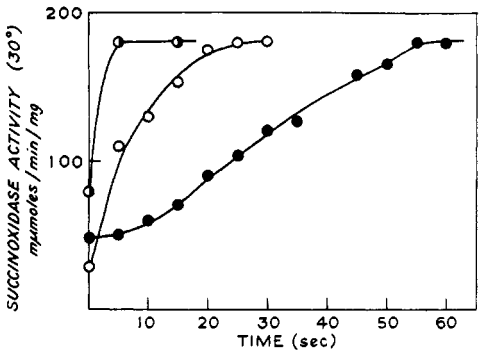

FIGURE 11: Effect of activation of succinate dehydrogenase on the succinoxidase activity of rat heart mitochondria in state 3. The mitochondria were suspended as in the experiments of Figure 4, brought to 30°, and 9 mM L-cysteinesulfinate was added to prevent accumulation of oxalacetate. The ordinate represents succinoxidase activity at 30°, measured polarographically, after the following treatments. Solid circles, 2 min at 30° with 0.34 mM ADP, then 15 mM succinate added; zero time indicates basal respiration before addition of substrate; half-filled circles, 1 min with 15 mM succinate, then 0.34 mM ADP added; zero time represents state-4 respiration immediately before succinate addition; open circles, 2 min with 3 mM pyruvate plus 3 mM malate in state 4, then piericidin A (0.62 mμmole/mg), succinate (15 mM), and ADP (0.34 mM) added simultaneously; zero time indicates state-4 respiration immediately before addition of succinate, ADP, and piericidin.

incubated with 0.34 mM ADP in the absence of substrate for 2 min at 30° and state 3 was initiated by the addition of 15 mM succinate (0 time). Full activation was reached in less than 1 min. The curve denoted with half-shaded circles represents an exiperiment in which the mitochondria were preincubated for 1 min at 30° with 15 mM succinate (no ADP, state 4) and 0.34 mM ADP was added at zero time. Full activation was observed within 5 sec, the dead time of the Oxygraph. The curve with open circles is an experiment in which the mitochondria were allowed to respire for 2 min in state 4 on pyruvate plus malate (3 mM each), then succinate (15 mM), ADP (0.34 mM), and piericidin A (0.62 mμmole/mg of protein) were added at 0 time, the latter to inhibit the oxidation of pyruvate plus malate. It may be seen that full activation was reached in much less time (20 sec) than in the first experiment, indicating that succinate dehydrogenase was extensively activated during the 2-min preincubation with NAD^+-linked substrates.

The experiments in Figure 11 illustrate another interesting feature of the regulation of succinate dehydrogenase in mitochondria. Full activation by succinate at 30° was much more rapidly attained than in membranal or soluble preparations under comparable conditions (Gutman *et al.*, 1971b; Kearney, 1957). Other experiments documented in this paper show similarly rapid activation by ATP or NAD^+-linked substrates (nearly full activation in 2–3 min). Not only does the rate of activation of the enzyme in mitochondria seem faster than in submitochondrial preparation but the activation energy of the process also appears to be lower in mitochondria. Full activation by pyruvate plus malate (in the presence of KCN) has been observed, for example, in 20 min at 15°, a temperature at which activation in soluble preparations appears to be exceedingly slow (Kearney, 1957). Although the energy of activation of the ATP-induced activation could not be accurately measured, possibly because of complications due to the temperature sensitivity of the adenine translocase system (Klingenberg and Pfaff, 1968), a preliminary estimate of the energy of activation for activation induced by pyruvate plus malate (10 kcal/mole) is much lower than the value obtained for membranal or soluble preparations (33–35 kcal/mole) (Gutman *et al.*, 1971b; Kearney, 1957). Possibly the conformational change in the enzyme, which is regarded as the basis of the activation (Kearney, 1957), is facilitated in the environment of the intact mitochondria.

Discussion

The experiments presented in this and the preceding papers in this series (Gutman *et al.*, 1971b) indicate that at least three types of positive modulation of succinate dehydrogenase exist in mammalian mitochondria. The first is activation by succinate or fumarate; the second activation by other substances which reduce the CoQ_{10} pool; and the third activation by ATP or a compound in equilibrium with it. Activation mediated by $CoQ_{10}H_2$ is most readily demonstrated in the presence of substrates (NAD^+-linked substrates, choline, and α-glycerophosphate) and cyanide, which prevents ATP accumulation. Neither this type of activation, nor that initiated by ATP appear to involve removal of inhibitory oxalacetate, for efficient transaminating agents do not block the ATP effect. Further, it has been pointed out that oxalacetate is separately compartmented from succinate dehydrogenase (Jones and Gutfreund, 1963). Furthermore, in our studies the addition of 0.2 mM oxalacetate to the heart mitochondria during activation did not interfere with activation by ATP or by pyruvate plus malate, nor was inhibition of succinate dehydrogenase detected in subsequent assays of aliquots, provided that succinate (15 mM) was present in the assay cuvets prior to disruption of the mitochondria with phospholipase A.

Deactivation in states 2 and 3 and in the presence of uncouplers is in accord with the known changes in the redox state of CoQ_{10} under these conditions (Kröger and Klingenberg, 1968). It should be noted, however, that (a) deactivation was far from complete in either state 2 or 3 in rat liver mitochondria and (b) that the more active the state-3 respiration, the less extensive was the deactivation of the dehydrogenase (Figure 3). It appears likely that in both situations ATP accumulation and consequent activation of the enzyme reverses the process, well before ADP is exhausted. Further, the negative correlation between respiration in state 3 and the extent of deactivation may mean that with the phosphorylating capacity limiting in rat liver mitochondria, the higher the electron flux from substrates to the respiratory chain, the less extensive is the oxidation of $CoQ_{10}H_2$ in state 3.

Despite indications in the literature that ATP might activate succinate dehydrogenase (*e.g.*, Gregolin and Scalella, 1965), the major effects noted here were unexpected. As already mentioned, there is no assurance that ATP as such reacts with the dehydrogenase and in so doing modulates it. One reason for this uncertainty is that ATP has no such effect in submitochondrial particles. The activations by ITP and IDP noted with membrane preparations do not necessarily bear a relation to ATP activation in mitochondria, partly because the concentrations required are quite different (μmolar concentration of ATP suffice for activation in mitochondria, mmolar concentrations of ITP and IDP are needed in ETP_H), partly because inosine nucleotides are not usually thought to be normal mitochondrial constituents, so that any speculation that ATP acts after conversion to ITP or IDP would seem premature. Unfortunately, activation by inosine nucleotides in mitochondria could not be tested directly, since they do not

penetrate. Nevertheless, the point seems established that purine di- and trinucleotides activate succinate dehydrogenase without intervention of the energy conservation system.

The three types of activators of succinate dehydrogenase which have been discovered may not be a complete list of physiological regulators of the enzyme. In recent, unpublished work succinyl-CoA was found to be an activator of the enzyme in soluble and membrane preparations. In occasional experiments activation by NAD^+-linked substrates or ATP, after reaching a maximum, reversed itself without an obvious reason, such as hydrolysis of ATP or oxidation of $CoQ_{10}H_2$. Although deactivation is a rapid and spontaneous process (Kimura *et al.*, 1967; Singer *et al.*, 1972), it is not inconceivable that negative modulators of the enzyme exist in mitochondria.

The data presented in this paper (particularly Figures 1 and 2) help explain several reports in the literature. Thus the steady-state concentration of succinate is lower (LaNoue *et al.*, 1970) and the labeling of malate by [^{14}C]succinate higher (Schäfer *et al.*, 1967; Von Korff, 1967; McElroy and Williams, 1968) in state 4 than state 3. This is in accord with the much higher succinate dehydrogenase activity in state 4 than in 3. Since uncouplers lead to deactivation of the dehydrogenase, this may explain the findings that uncouplers prevent the reduction of CoQ_{10} by succinate in the presence of antimycin (Kröger and Klingenberg, 1966) and cause succinate accumulation (Tsuiki *et al.*, 1968). Reversible activation of succinate dehydrogenase may also provide an alternate explanation, not involving oxalacetate removal, for the frequently noted effect of ATP in increasing the rate of succinate oxidation in aged or ADP-inhibited pigeon heart mitochondria (Chance and Hagihara, 1962) and in arsenate-treated liver mitochondria (Azzone and Ernster, 1960), and for the reactivation of succinate oxidation in aged mitochondria by NAD^+-linked substrates (Schollmeyer and Klingenberg, 1961). It also provides a mechanism for the crossover between succinate and malate noted by Schäffer *et al.* (1967) and Von Korff (1967) in the state $4 \rightarrow 3 \rightarrow 4$ transitions.

The allosteric regulation of succinate dehydrogenase (Kearney, 1957; Gutman *et al.*, 1971b; Singer *et al.*, 1972) offers a firm basis for occasional suggestions in the literature that the succinate–fumarate step may be an important regulatory site in the Krebs cycle (Williams, 1965; Von Korff, 1967). In considering the physiological purpose of this regulation, it may be recalled that succinate dehydrogenase activity is high in the controlled state where ATP concentration is high and is constrained in the active state where ATP concentration is low and, therefore, synthesis of ATP is called for.

Another relevant fact, known for many years, to be considered is that electron flux from the various respiratory chain-linked flavoproteins to the electron-transport system occurs in a competitive manner, *i.e.*, the oxidation of succinate inhibits that of NADH, choline, etc., and *vice versa* (Wu and Tsou, 1955; Kimura and Singer, 1959; Ringler and Singer, 1958, 1959). In fact, the simultaneous oxidation of two substrates may yield a rate of O_2 uptake which is less than that observed with the faster substrate alone.

It is also known that the succinate dehydrogenase activity of many mammalian cells is well in excess of the electron-carrying capacity of the respiratory chain and of the phosphorylating capacity of the energy conservation system (Singer, 1966). It seems very likely, therefore, that unconstrained succinate dehydrogenase activity, oxidizing succinate derived from α-ketoglutarate, odd-numbered fatty acids, and branched amino acids, would inhibit NADH oxidation. This would, in turn, lower the rate of ATP synthesis, since while succinate oxidation yields only 2 moles of ATP in each turn of the citric acid cycle, NADH oxidation yields 9 by oxidative phosphorylation and a 10th arises from substrate level phosphorylation.

It is therefore proposed that when the ATP:ADP ratio is low (state 3) deactivation of succinate dehydrogenase is of benefit to the cell since it permits faster NADH oxidation and, hence, ATP synthesis. Under these conditions succinate accumulates (Schäfer *et al.*, 1967; Von Korff, 1967; LaNoue *et al.*, 1970). The onset of ATP synthesis provides an activator of the dehydrogenase, so that ATP prevents deactivation from becoming extensive enough for succinate dehydrogenase to become seriously rate limiting in the operation of the cycle. As state-4 conditions are approached, the dehydrogenase becomes activated again, in part by the accumulated ATP and succinate, in part by the gradual reduction of CoQ_{10}. With the constraint removed, the dehydrogenase can then oxidize the accumulated succinate. Thus a constant fluctuation is pictured as occurring in the mitochondrion between metabolic conditions approaching state 4 and state 3, respectively, although the mitochondrial population in intact tissue may yield an average value between states 3 and 4, as has been reported for heart (Chance *et al.*, 1965).

Further details of this hypothesis and alternative explanations of the intricate, multiple regulation of succinate dehydrogenase will be given elsewhere (Singer *et al.*, 1971, 1972).

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Chromatographic Separation of Chick Brain Chromatin Proteins Using a SP-Sephadex Column*

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ABSTRACT: Salt-dissociated chromatin proteins isolated from 11-day-old chick embryo brain tissue were fractionated by chromatography on SP-Sephadex columns using a step sodium chloride gradient. The column was equilibrated in 7 M urea–0.23 M NaCl–0.01 M NaAc buffer at pH 5.2. Total recovery of protein from the column averaged approximately 90%. At least 90% of the total nonhistone proteins of chromatin was eluted from the column at 0.23 M NaCl; histone IIb_1 at 0.3 M NaCl; histones IIb_2, III, and IV at 0.4 M NaCl; and histone I at 0.8 M NaCl. The 10% or so of the nonhistone proteins which were not eluted by 0.23 M NaCl were recovered partially at 0.3 M NaCl and partially at 0.4 M NaCl. These nonhistone proteins eluted at 0.3 and 0.4 M NaCl were also present in the group of proteins eluted at 0.23 M NaCl. Thus the latter is a representative population of all nonhistone proteins. The molecular weight of the smallest nonhistone protein was found to be *ca.* 10,000. The largest nonhistone protein had molecular weight greater than *ca.* 200,000.

Chromatin isolated from higher organisms is composed of DNA, RNA, and protein, the protein complement consisting of histones and nonhistones. The amount of nonhistone protein found in the chromatin varies with the organism (Bonner *et al.*, 1968). The amount of chromosomal nonhistone protein found also varies within tissues of the same organism (Dingman and Sporn, 1964) and with the developmental stage of the organism (Marushige and Ozaki, 1967). In general, chromatin obtained from metabolically active tissues contains a greater quantity of nonhistone protein than chromatin obtained from inactive tissues (Seligy and Miyagi, 1969; Paoletti and Huang, 1969). The functional importance of these proteins in controlling the template activity of chromatin has been indicated in several studies (Wang, 1969; Gilmour and Paul, 1970; Spelsberg and Hnilica, 1969; Teng *et al.*, 1971). There are, however, no conclusive studies in regard to the role of these proteins in chromatin structure. Attempts to understand the role of the nonhistone proteins in the organization of the genome will depend largely upon the successful isolation of these proteins, free of histones and in good yield.

There are two principle approaches to the isolation of nonhistones from chromatin. One method employs an initial extraction of histones by strong acids (HCl or H_2SO_4), followed by solubilization of the nucleic acid and nonhistones in detergent (Marushige *et al.*, 1968), phenol (Shelton and Allfrey, 1970; Shelton and Neelin, 1971; Teng *et al.*, 1971), or concentrated salt solution of high pH (Benjamin and Gelhorn, 1968). Subsequently, the nucleic acid and nonhistone are separated by centrifugation according to their difference in molecular weight (Marushige *et al.*, 1968) or density (Benjamin and Gelhorn, 1968). Alternatively, the nucleoprotein complex can be partitioned between aqueous buffers and phenol (Shelton and Neelin, 1971; Teng *et al.*, 1971). Using

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