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NATURE OF THE ACTIVATION OF SUCCINATE DEHYDROGENASE BY VARIOUS EFFECTORS AND IN HYPOBARIA AND HYPOXIA

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SUMMARY

1. Hepatic mitochondrial succinate dehydrogenase (succinate:(acceptor)oxidoreductase, EC 1.3.99.1) was activated by preincubation of mitochondria with four diverse classes of compounds, the dicarboxylic acids, nitrophenols, quinols (and ubiquinols) and pyrophosphates. Of the various compounds tested malonate, oxaloacetate and pyrophosphate, well-known competitive inhibitors of the enzyme, and also hydroquinone and ubiquinols were effective even at low concentrations and showed maximal stimulation in 2 min.

2. Activation of succinate dehydrogenase by ubiquinol-9 and ubiquinol-10 was comparable to succinate activation in fresh mitochondria, and was much higher in the aged samples.

3. Preincubation of mitochondria with succinate, 2,4-dinitrophenol, pyrophosphate and ATP also stimulated the succinate–2,2′,5,5′-tetraphenyl-3,3′-(4,4′-biphenylene) ditetrazolium chloride (NT) reductase activity, whereas malonate, hydroquinone and ubiquinol-9 were ineffective. A differential activation of the flavoprotein by the oxidized and reduced forms of ubiquinone-9 was observed, the former stimulating the reduction of NT and the latter of phenazine methosulphate–2,6-dichlorophenolindophenol.

4. Repeated washing of the activated mitochondrial samples with the sucrose homogenizing medium, partially reversed the activation by effectors other than succinate. Further washing of the activated preparations after a second preincubation with succinate reverted the enzyme activity to the basal level in the case of malonate, ATP and pyrophosphate but not that of hydroquinone and ubiquinol-9.

5. Increase in the activity of hepatic mitochondrial succinate dehydrogenase, but not of succinate–NT reductase, known to occur in rats exposed to hypobaria was also observed in hypoxia indicating that it is an effect of lowered O_2 tension. The enzyme activity in these "partially activated" preparations was stable to washing with the sucrose homogenizing medium and could be fully activated to the same level as in the controls showing thereby the qualitative nature of the change. On washing these succinate-activated preparations further with the medium, the "hypobaric activation" was not reversed to the basal level, whereas the "hypoxic activation" was reversed. These results suggest that the effectors responsible for the activation

Abbreviations: NT, 2,2′,5,5′-tetraphenyl-3,3′-(4,4′-biphenylene) ditetrazolium chloride; PMS, phenazine methosulphate; DCIP, 2,6-dichlorophenolindophenol.

of succinate dehydrogenase under hypobaric and hypoxic conditions are probably different; the former may be of the ubiquinol type and the latter of the malonate type.

INTRODUCTION

Mitochondrial succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) is known to undergo a reversible activation on preincubation with succinate, malonate and other compounds capable of combining at the active site[1,2]. It was also observed in this laboratory that the hepatic mitochondrial succinate dehydrogenase activity increased in rats exposed to hypobaric conditions[3]. This effect was also observed in animals exposed to a low partial pressure of O_2 and hence appeared to be an effect of lowered O_2 tension. This increased activity in succinate dehydrogenase obtained under hypoxic conditions differed from that of succinate activation in being stable to washing with the sucrose homogenizing medium. It appeared that the natural effector under hypobaric and hypoxic conditions may be bound strongly to the enzyme as in the case of malonate. This prompted a survey of various types of activators and the properties of the activated preparations hopefully, to gain insight into the mechanism of hypobaric and hypoxic induced activation phenomena.

Recently, it has been shown that 2,4-dinitrophenol, a competitive inhibitor of succinate dehydrogenase[4], the reduced form of ubiquinone-10 (ref. 5) and ATP (ref. 6) could also activate the enzyme on preincubation with mitochondria. Preliminary studies from this laboratory showed that the activation phenomenon was not specific to ubiquinol-9 (ref. 7), the natural, major homologue present in rat liver mitochondria, since the other homologues of reduced ubiquinone and other quinols like plastoquinol-9 and hydroquinone were also found to be effective activators of the enzyme activity.

In view of the diversity of the effectors that can activate succinate dehydrogenase, namely, the dicarboxylic acids, quinols, ubiquinols, nitrophenols and pyrophosphates, it was of interest to study the action of each type of activator with respect to the specificity, optimal concentration for maximal stimulation, and the reversibility of the activation on washing with the sucrose homogenizing medium.

It is generally considered that the determination of succinate–2,2′,5,5′-tetraphenyl-3,3′-(4,4′-biphenylene) ditetrazolium chloride (NT) reductase activity would also give a measure of the succinate dehydrogenase activity. This activity could also be stimulated by preincubation with succinate[8] although the activation obtained is only marginal as compared to that in the phenazine methosulphate (PMS)–2,6-dichlorophenolindophenol (DCIP) assay. Hence it was of interest to study the effect of these activators on the succinate–NT reductase activity also with a view to compare these effects with those observed under hypobaric and hypoxic conditions. A detailed account of this work is presented in this communication.

EXPERIMENTAL

Homologues of ubiquinone (9 and 10) were received as gifts from Hoffmann–La Roche and Co, Ltd, Basle (Switzerland). Phenazine methosulphate was purchased

from Calbiochem, Inc, Los Angeles, Calif. (U.S.A.). All other chemicals used were of Analar grade. The reduced forms of the homologues of ubiquinone were prepared as described by Ramasarma and Lester[9].

Hypobaric chamber

Male albino rats (weighing 150–170 g) from the stock colony were exposed to an atmospheric pressure of 350±5 mmHg, corresponding to an altitude of about 6100 m, in a decompression chamber fabricated in this laboratory[10]. This is an open-circuit chamber where the exhaled CO_2 is flushed out along with the air. This chamber provides conditions of both low pressure and low O_2 tension. Low pressure was obtained in the chamber in less than 3 min after the start of the evacuation and was maintained for 4 h. At the end of this period, atmospheric pressure was restored, the animals were removed and killed by stunning and decapitation. The livers were removed and processed further.

Hypoxia chamber

The instrument was designed to have a gas mixture of known composition to flow through a chamber. This arrangement facilitated conducting experiments on the exposure of animals to hypoxia for long periods, whereby low O_2 tension could be maintained at one atmospheric pressure and the exhaled CO_2 flushed out of the chamber. Cylinders of N_2 and O_2 with regulating valves were connected to flow meters through stopcocks for fine control of the flow rates. The gases coming off the flow meters at constant rates were mixed in a jar plugged with cotton and passed through an air-tight chamber where the animals were placed, and let out through an outlet at the top. In the present experiments, 10% O_2 and 90% N_2 mixtures were used which gave a partial pressure of O_2 equivalent to about half the atmospheric pressure at Bangalore (altitude, 914 m above mean sea level).

Preparation of rat liver mitochondria

The method followed was the same as described previously[4].

Enzyme assays

Succinate dehydrogenase activity was determined spectrophotometrically with PMS and DCIP as the electron acceptor system as described by Arrigoni and Singer[11]. The details of the assay system are as described in a previous paper[4].

Succinate–NT reductase activity in mitochondria was determined by measuring the formazan produced on enzymic reduction of NT (ref. 12). The reaction mixture consisted of 70 μmoles of potassium phosphate buffer (pH 7.4), 50 μmoles of succinate, 1.5 μmoles of NT, 0.6 μmole of KCN (freshly neutralized) and about 1 mg of mitochondrial protein in a total volume of 1.1 ml. The reaction mixture was incubated at 37 °C for 10 min and then 1 ml of 10% trichloroacetic acid was added. The formazan produced was extracted with 5 ml of ethyl acetate and measured by its $A_{510\,nm}$. The enzyme activities were expressed as munits per mg of protein, one unit being 1 μmole of dye reduced/min. Unless otherwise stated, all enzyme activities were determined at 25 °C. Protein was determined by the biuret method[13].

Preincubation of mitochondria for activation

Preincubations were carried at 37 °C, or at 30 °C where specified, for the

periods indicated. The preincubation mixture contained about 2–5 mg of mitochondrial protein, 100 μmoles of potassium phosphate buffer (pH 7.6) and 50 μmoles of succinate, or the compounds at the concentrations indicated, in a total volume of 1 ml. An aliquot of this mixture was used for assaying the enzyme activity. The activities determined after preincubating the mitochondrial samples with 100 μmoles of potassium phosphate buffer (pH 7.6) served as the control values.

"Washing" the activated preparations

The procedure followed for washing the activated mitochondrial samples was the same as described previously[4]. In these experiments about 25–40 mg of the mitochondrial protein were used. The reversibility of the activity on washing the activated preparations was always compared with the "succinate-activated" preparation treated similarly, in order to determine whether these effectors bind firmly to the enzyme or could be easily removed on "washing" as shown in the case of the binding of succinate[14]. A second preincubation of these twice-washed preparations with succinate, followed by two more washings, was carried out to study whether these firmly bound activators could be displaced by succinate, resulting in reversal of the enzyme activity to the basal level.

RESULTS

The four main classes of compounds studied as activators of succinate dehydrogenase were the organic acids, nitrophenols, quinols and ubiquinols, and pyrophosphates. The activation phenomenon was studied with respect to the optimal concentration, kinetics of activation and reversibility of the activation on washing with the sucrose homogenizing medium. Also, the effect of preincubation of mitochondria with these compounds on the succinate–NT reductase activity was studied, choosing a few compounds representative of each group of activators.

Activation by organic acids

In the first set of experiments, the effect of preincubating mitochondria with various organic acids was studied and the results are shown in Table I. A comparison of the activation obtained by preincubation with mono-, di- and tricarboxylates showed that dicarboxylates were the most effective. Oxalate, malonate and succinate caused a maximal activation of 78–82% over the control, while pyruvate and citrate gave only a marginal stimulation of 20 and 48%, respectively. The other dicarboxylates tested, malate and α-oxoglutarate, showed no activation. It is interesting to note that oxaloacetate, a powerful competitive inhibitor of succinate dehydrogenase, could also activate the enzyme on preincubation at a very low concentration of 50 μM. A similar activation has been observed with other competitive inhibitors like malonate[2], 2,4-dinitrophenol[4] and pyrophosphate.

While in the case of succinate, a concentration of 5 mM was required for maximal activation, malonate was efficient as an activator even at a concentration two orders of magnitude less than that of succinate. A comparison of the time course of activation by succinate and malonate indicated that the activation by the latter was very fast being maximal in 2 min at 37 °C. Activation by succinate was slow

and required about 6 min for attaining the maximal activity which is in agreement with the earlier studies by Kimura *et al.*[14].

TABLE I

ACTIVATION OF SUCCINATE DEHYDROGENASE BY SOME ORGANIC ACIDS

Mitochondria (about 3 mg of protein) were preincubated at 37 °C with the compounds indicated for 7 min, this period being sufficient for attaining maximal activation. No stimulation was observed when these compounds at the concentrations indicated were added to the assay system and the enzyme activity assayed without preincubation.

Additions in the preincubation medium		*Enzyme activity (munits/mg of protein)*	*% stimulation (+) or % inhibition (−)*
Compound	*mM*		
None		65	
Monocarboxylate			
Pyruvate	5.0	78	20 (+)
Dicarboxylate			
Oxalate	5.0	118	82 (+)
Malonate	0.1	116	78 (+)
Methyl malonate	0.1	91	40 (+)
Succinate	50.0	118	82 (+)
Oxaloacetate	0.05	89	37 (+)
Malate	2.5	32	50 (−)
α-Ketoglutarate	5.0	65	Nil
Tricarboxylate			
Citrate	5.0	96	48 (+)

Nitrophenols and quinols as activators of succinate dehydrogenase

The second group of compounds which were capable of stimulating the succinate dehydrogenase activity were the nitrophenols and quinols (Table II). Of the three nitrophenols tested, 2,4-dinitrophenol, a competitive inhibitor was most effective as an activator of succinate dehydrogenase. 2-Nitrophenol and 4-nitrophenol showed only small stimulatory effects. On the other hand, hydroquinone at a very low concentration gave a large stimulation.

A time course of this activation indicated that the activation obtained with hydroquinone was extremely fast, reaching a maximum in 2 min at 37 °C (Fig. 1). Further preincubation of mitochondria beyond 2 min resulted in a decrease in the enzyme activity, presumably due to inactivation of the enzyme. In view of the fast activation at 37 °C the preincubation was done at 30 °C. The results shown in Fig. 1 indicated that even at this temperature the rate of activation was fast and the maximal activity was reached in 10 min.

Activation of succinate dehydrogenase by ubiquinols in fresh and aged mitochondrial samples

Studies by Gutman *et al.*[5,6] showed that succinate dehydrogenase was activated by ubiquinol-10 in intact mitochondria. Our studies indicated that at a concentration

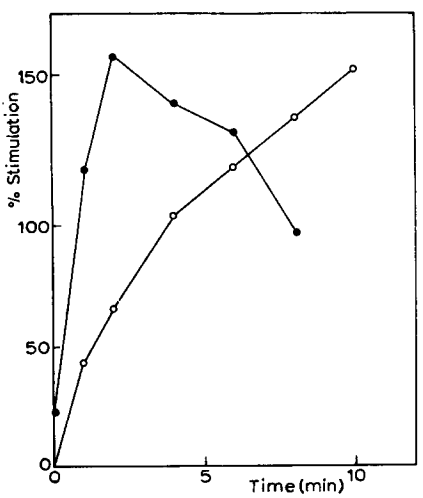
TABLE II

ACTIVATION OF SUCCINATE DEHYDROGENASE BY NITROPHENOLS AND QUINOLS

Mitochondria (about 3 mg of protein) were preincubated at 37 °C with the compounds indicated. In the case of quinols, antimycin A (3 μg) was included in the preincubation mixture. The preincubation was carried out for 7 min with nitrophenols and for 2 min with quinols, these periods being sufficient for maximal activation as indicated by preliminary experiments. No stimulation was observed when these compounds at the concentrations indicated were added to the assay system and the enzyme activity assayed without preincubation.

Additions in the preincubation medium		*Activity (munits/mg of protein)*	*% stimulation*
Compound	*mM*		
None		68*	
Nitrophenols			
2,4-Dinitrophenol	7.0	97	47
2-Nitrophenol	7.0	87	32
4-Nitrophenol	7.0	79	19
Quinols			
Catechol	0.09	89	31
Resorcinol	0.09	83	22
Hydroquinone	0.09	129	90

* Preincubation with succinate (50 mM) for 7 min at 37 °C gave an activity of 113 (66%).



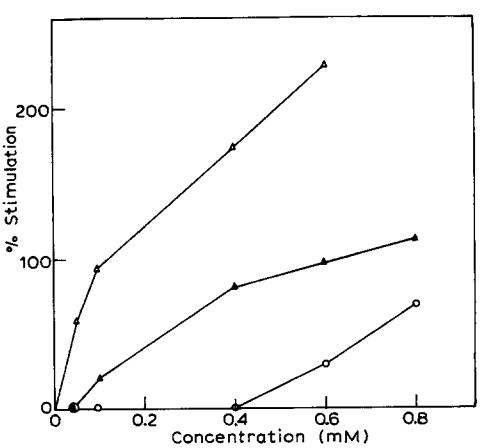


Fig. 1. Time course of the activation of succinate dehydrogenase by hydroquinone. Mitochondria (about 2 mg of protein) were preincubated at 37 °C (●) and 30 °C (○) for the periods indicated, with 100 mM phosphate buffer and 90 μM hydroquinone.

Fig. 2. Activation of succinate dehydrogenase in fresh and aged mitochondria by ubiquinol-9. Freshly prepared mitochondria (about 3 mg of protein) were preincubated at 37 °C for 2 min with 100 mM phosphate buffer, antimycin A (2 μg) and ubiquinol-9 (▲) as shown in the figure. A similar experiment was carried out without the addition of antimycin A (○) in the preincubation medium. The mitochondrial sample was stored frozen for 5 days and the experiment with antimycin A was repeated with this aged sample (△). The control values for the fresh and aged mitochondrial samples were 47 and 63, respectively. Preincubation of mitochondria with 50 mM succinate gave a stimulation over the control of 119% with the fresh preparation and 91% with the aged sample.

of 0.6 mM, ubiquinol-9 gave a stimulation of 95% over the control when preincubated with a freshly isolated mitochondrial sample and a much higher stimulation of 230% when the experiment was repeated using the same mitochondrial sample after aging for 5 days (Fig. 2). The increase was true even when compared on activity basis and not a mere reflection of decreased basal activity. The data in Fig. 2 also indicate that it was necessary to keep the ubiquinone-9 in the reduced form, since omission of antimycin A in the preincubation medium which resulted in oxidation of added ubiquinols did not show the stimulation of the enzyme activity. However, at higher concentrations of ubiquinol, a small stimulatory effect was observed even in the absence of added antimycin A presumably due to the residual ubiquinol-9 present in the preincubation mixture, since only a short period of 2 min was used for the preincubation.

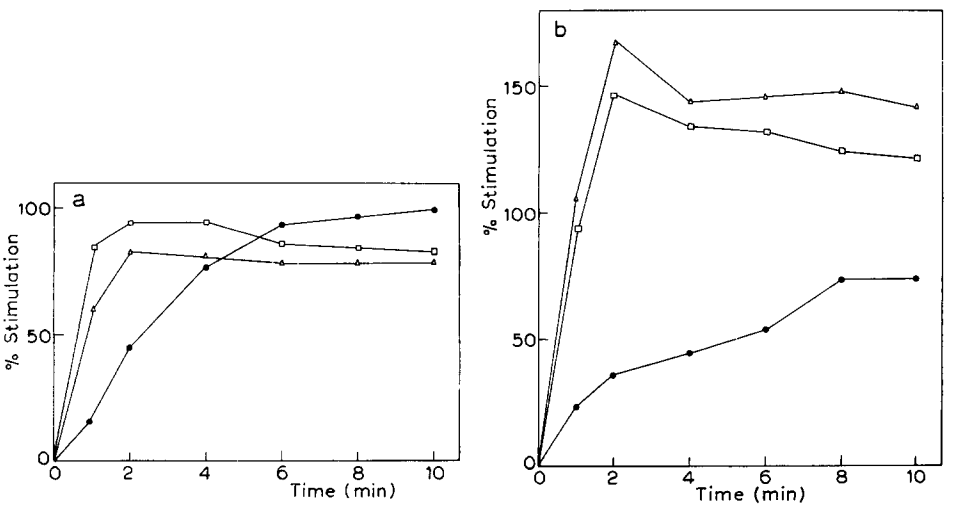


Fig. 3. (a) Time course of the activation of succinate dehydrogenase by ubiquinol-9 and ubiquinol-10 in fresh mitochondria. Freshly prepared mitochondria (4 mg of protein) were preincubated at 37 °C with 100 mM phosphate buffer, antimycin A (4 μg) and ubiquinol-9 (△) or ubiquinol-10 (□). For comparison a similar experiment with 50 mM succinate (●) was carried out at 37 °C. (b) Activation of succinate dehydrogenase in aged mitochondrial preparations by ubiquinol-9 and ubiquinol-10. The same mitochondrial sample as in (a) was used after storing frozen for 5 days. The experimental conditions observed in the case of succinate (●), ubiquinol-9 (△) and ubiquinol-10 (□) were as given in the legend for (a).

A time course of the activation of succinate dehydrogenase activity in fresh mitochondria (Fig. 3a) and in aged preparations (Fig. 3b) clearly showed that both ubiquinol-9 and ubiquinol-10 were efficient activators. The stimulation of activity brought about by both these compounds in fresh mitochondria was comparable to that of succinate. However, the results with the aged preparations (Fig. 3b) presented a completely different picture wherein the stimulation by ubiquinols was much higher than that of succinate. One possible explanation is that the ubiquinols may be more readily permeable in aged mitochondrial preparations than in the fresh samples. But the significance of the high activation by lipid quinols in aged mitochondria is not clear.

Enhancement of succinate dehydrogenase activity by pyrophosphates

Activation by ATP of succinate oxidation in yeast mitochondria was first observed by Gregolin and Scalella[15], who interpreted it as a direct effect and not by removal of an inhibitor. Studies by Gutman *et al.*[6] with intact rat heart mitochondria showed an oligomycin-insensitive activation of succinate dehydrogenase by ATP. Ths results presented in Table III show that apart from ATP and ADP, pyrophosphate can also stimulate the enzyme activity. Of these, pyrophosphate was the most effective. AMP did not show any such effect. This presumably means that the pyrophosphate moiety somehow is involved in the binding of these effectors to the enzyme.

TABLE III

ACTIVATION OF SUCCINATE DEHYDROGENASE ON PREINCUBATING MITOCHONDRIA WITH PYROPHOSPHATES

Mitochondria (about 4 mg of protein) were preincubated at 37 °C with the compounds indicated for 7 min. No stimulation was observed when 0.5 mM ADP was added in the assay system and the enzyme activity determined without preincubation, whereas ATP and sodium pyrophosphate at this concentration in the assay gave a stimulation of 45 and 38%, respectively.

Additions in the preincubation medium		*Activity (munits/mg of protein*	*% stimulation (+) or % inhibition (−)*
Compound	*mM*		
None		40*	
AMP	0.5	39	3 (−)
ADP	0.5	68	70 (+)
ATP	0.5	73	83 (+)
Pyrophosphate	0.1	93	133 (+)

* Preincubation with succinate (50 mM) gave an activity of 97 (143%).

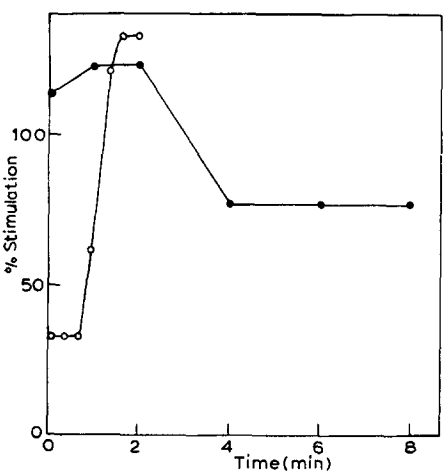


Fig. 4. Time course of the stimulation of succinate dehydrogenase activity by pyrophosphate. Mitochondria (about 3 mg of protein) were preincubated with 100 mM phosphate buffer and 0.1 mM sodium pyrophosphate at 37 °C (●) and 30 °C (○) for the time intervals indicated. The enzyme activities were assayed at 25 °C in the former case and at 15 °C in the latter.

It is of interest to note that pyrophosphate which acts as a powerful competitive inhibitor of succinate dehydrogenase at higher concentrations[16], activates the enzyme at lower concentrations, thus resembling malonate, oxaloacetate and 2,4-dinitrophenol.

At a concentration of 0.1 mM, the stimulation produced by pyrophosphate was maximal. Concentrations above this were inhibitory probably due to the inhibitory effect of higher concentrations of pyrophosphate transferred from the preincubation mixture to the assay.

A time-course study of the activation by pyrophosphate showed that it was very fast at 37 °C being 113% above the basal activity even at "zero time" despite short periods of assay (Fig. 4). Owing to the rapid activation observed at 37 °C, the preincubation was carried out at 30 °C and the enzyme activity assayed at 15 °C to avoid any further activation in the assay. The results indicated that there was an appreciable activation of the enzyme activity even at 30 °C and the maximal activity was reached within 2 min (Fig. 4).

Effect on succinate–NT reductase activity

The effect of preincubating mitochondria with these compounds on the succinate–NT reductase activity is shown in Table IV. The data indicated that, unlike succinate, malonate, citrate and quinols did not stimulate the activity despite being efficient activators of the succinate dehydrogenase activity as determined by the PMS–DCIP assay. On the other hand, the nitrophenols and the pyrophosphate did show appreciable stimulation of this enzyme activity.

Succinate–NT reductase activity has been shown by Lester and Smith[17] to be dependent on ubiquinone and hence may function as a shunt pathway in the electron transport system. The results presented in Table IV show that succinate–NT reductase activity is enhanced to a great extent on preincubation of mitochondria with ubiquinone-9, whereas ubiquinol-9 and other quinols were ineffective. This is interesting in view of the fact that the oxidized and reduced forms of ubiquinone appear to activate two different activities of the same flavoprotein, the former enhancing the succinate–NT reductase activity and the latter the succinate–PMS–DCIP reductase activity.

Reversibility of the activated preparations on washing

Studies by Kimura *et al.*[14] have shown that succinate activation could be reversed on removing the bound effector by repeated washing of the activated mitochondrial preparations with the sucrose homogenizing medium. A similar treatment carried out with the activated preparations obtained on preincubating mitochondria with the various effectors resulted only in a partial reversal of the activation (Table V) showing thereby that these effectors may be firmly bound to the enzyme protein. Nevertheless, a second preincubation of these twice-washed preparations with 50 mM succinate, followed by two more washings with the sucrose homogenizing medium reversed the activity to the basal level in the case of malonate, ATP and pyrophosphate. However, the hydroquinone- and ubiquinol-activated preparations could only be partially reversed and still retained the enzyme activity at 43 and 105% above the basal level, respectively.

TABLE IV

EFFECT OF PREINCUBATING MITOCHONDRIA WITH THE EFFECTORS ON SUCCINATE–NT REDUCTASE ACTIVITY

The enzyme activities were determined after preincubating mitochondria (about 2 mg protein) with the compounds at 37 °C for the periods indicated. In the case of quinols and ubiquinol-9 0.6 mM KCN (freshly neutralized) was included in the preincubation medium to prevent oxidation, since antimycin A is known to inhibit the succinate–NT reductase activity[17].

Preincubation conditions			*Activity munits/mg of protein*	*% stimulation (+) or % inhibition (−)*
Compound	*Concentration (mM)*	*Time (min)*		
I. *Organic acids*				
None		7	7.8	
Succinate	50.0	7	10.9	40 (+)
Malonate	0.1	7	8.2	5 (+)
Citrate	5.0	7	8.2	5 (+)
II. *Nitrophenols*				
None		7	7.8	
2,4-Dinitrophenol	2.0	7	10.7	37 (+)
2-Nitrophenol	7.0	7	10.5	35 (+)
4-Nitrophenol	7.0	7	8.3	6 (+)
III. *Quinols and quinones*				
None		7	8.8	
Catechol	0.09	2	9.0	2 (+)
Hydroquinone	0.09	2	9.0	2 (+)
Ubiquinol-9	0.47	2	7.7	12 (−)
Ubiquinone-9	0.47	7	22.2	152 (+)
IV. *Pyrophosphates*				
None		7	7.8	
ATP	0.5	7	12.0	54 (+)
Pyrophosphate	0.1	7	12.7	63 (+)

TABLE V

REVERSIBILITY OF ACTIVATION OF SUCCINATE DEHYDROGENASE ON WASHING OF MITOCHONDRIA

Mitochondria (25–50 mg of protein) were preincubated at 37 °C as indicated below. In the case of hydroquinone and ubiquinol-9, antimycin A (1 μg/mg protein) was added in the preincubation medium.

Preincubation conditions			*Activity, % of controls*			
			1st preincubation		*2nd preincubation*	
Compounds	*Concentration (mM)*	*Time (min)*	*Initial*	*Washed twice*	*Initial*	*Washed twice*
Succinate	50.0	7	181	89	—	—
Malonate	0.1	7	199	198	230	93
Hydroquinone	0.1	2	212	160	200	143
Ubiquinol-9	0.5	2	262	192	302	205
ATP	0.5	7	211	133	198	76
Pyrophosphate	0.1	7	187	153	193	113

Increase of succinate dehydrogenase activity in hypobaric and hypoxic conditions

Previous work from this laboratory showed that hepatic mitochondrial succinate dehydrogenase activity increased in rats exposed to low atmospheric pressure (350 ± 5 mmHg) for short periods[3]. This effect was also observed in rats exposed to a low partial pressure of O_2 (equivalent to that at about half atmospheric pressure) for 4 h (Table VI). These results indicated that the increased activity of succinate dehydrogenase was probably an effect of lowered O_2 tension prevalent under both hypobaric and hypoxic conditions. Also, these partially activated preparations obtained under hypobaria and hypoxia could be fully activated by preincubation at 37 °C with succinate for 7 min. The extent of activation was the same in both the controls and

TABLE VI

ACTIVATION OF SUCCINATE DEHYDROGENASE ON BRIEF EXPOSURE TO HYPOBARIC AND HYPOXIC CONDITIONS

Rats were exposed to half-atmospheric pressure or to low O_2 tension for 4 h and the hepatic mitochondrial fractions were obtained as described in the text. Succinate dehydrogenase and succinate–NT reductase activities were determined with and without preincubation with succinate. The values represent the mean ± S.D. of samples processed from six animals in each group. The figures in parentheses indicate the percent of the control value.

Conditions	*Control*	*Hypobaric*	*Hypoxic*
I. *Succinate–PMS–DCIP reductase activity*			
Basal	40 ± 3 (100)	64 ± 12 (160)	69 ± 9 (173)
		$P < 0.01$	$P < 0.01$
Preincubated with succinate	127 ± 9 (100)	123 ± 11* (97)	138 ± 14* (109)
II. *Succinate–NT reductase activity*			
Basal	11.4 ± 1.6 (100)	12.4 ± 3.2* (109)	12.2 ± 1.9* (107)
Preincubated with succinate	14.0 ± 1.6 (100)	15.2 ± 1.7* (108)	15.7 ± 1.2* (112)

* Statistically not significant.

TABLE VII

EFFECTS OF WASHING OF MITOCHONDRIA BEFORE AND AFTER PREINCUBATION WITH SUCCINATE ON THE SUCCINATE DEHYDROGENASE ACTIVITY IN THE HYPOBARIC AND HYPOXIC SAMPLES

Mitochondria (about 40 mg of protein) from the control and the exposed animals were used for this experiment. Preincubation after washing was carried out at 37 °C for 7 min with 50 mM succinate. The values represent the mean of samples processed from six animals in each group. The figures in parentheses indicate the percentage activity of the respective control value.

Conditions	*Control*	*Hypobaric*	*Hypoxic*
Initial	57 (100)	99 (173)	100 (175)
Washed twice with the homogenizing medium	45 (100)	87 (193)	82 (182)
Preincubated with succinate after washing	118 (100)	163 (152)	134 (114)
Washed twice with the homogenizing medium after preincubation	47 (100)	86 (183)	55 (117)

the exposed animals indicating thereby the qualitative nature of the change. However, exposure of rats to hypobaric and hypoxic conditions did not increase the succinate–NT reductase activity (Table VI).

Washing the activated mitochondrial preparations obtained under hypobaric and hypoxic conditions (Table VII) with the sucrose homogenizing medium showed that the enhanced enzyme activity was stable to washing being 50–60% above the basal activity, unlike the succinate-activated preparations. These partially activated preparations obtained after washing could be further activated by preincubating with succinate. Rewashing of these fully activated preparations reverted the activity to the basal level in the case of hypoxia whereas in the case of hypobaria, the enzyme activity could not be reverted to the basal level but remained at the partially activated state. It can be concluded from these observations that the effectors responsible for the activation of succinate dehydrogenase under hypobaric and hypoxic conditions are probably different.

DISCUSSION

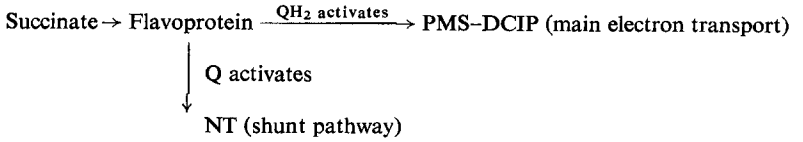
The bewildering diversity in the nature of the compounds that are capable of activating mitochondrial succinate dehydrogenase makes it difficult to understand the underlying mechanism. The compounds fall into the classes of organic acids, nitrophenols, quinols and pyrophosphates. The one common requirement for the activation is the preincubation of mitochondria with these compounds. Neither oxidation nor any other reaction of the compounds seems to occur. Only a simple, physical, temperature-dependent association of the effector with the enzyme seems to be the basic feature of this phenomenon.

On a close scrutiny of the structural aspects of these compounds a common feature became apparent. This is the presence of two ionizable oxygen atoms spatially separated. The distance between the two is about 7 Å in ubiquinol and other quinols with little possible deviation, restricted by the benzene ring as judged by the Courtauld space-filling models. Other activating compounds also are capable of providing two oxygens at such a distance, including succinate. These probably facilitate the appropriate interaction with specific sites on the protein, presumably two positively charged groups which on neutralization may result in increased molecular capacity of the enzyme.

An interesting feature observed in the present specificity studies was the differential activation with respect to the reduction of PMS–DCIP and NT. While the nitrophenols and pyrophosphates were capable of showing activation in both assays, malonate, ubiquinol and compounds related to them were found to be effective with the reduction of PMS–DCIP and not of NT. In view of the apparent similarities of the structural features of the effectors and the sites on the protein, this observation is difficult to reconcile unless the possibility of differential modification of the dye-acceptor sites is involved. Such a role may be played by the iron–sulphur proteins found to be associated with succinate dehydrogenase[18].

The precise nature of the sites of NT reduction by succinate dehydrogenase is obscure[17]. However, this enzyme activity is known to function as a slower shunt pathway and some evidence is available for its role in heat generation[8]. It is noteworthy that ubiquinone has a dual role to play in the activation of this enzyme

system with remarkable specificity. In the reduced form it activates the main electron transport and in the oxidized form the shunt pathway is activated as represented schematically below:



The regulation of succinate dehydrogenase by the redox status of ubiquinone in relation to NADH oxidation and ATP/ADP ratio had been extensively discussed by Gutman *et al.*[6] and its role in the electron transport by Singer *et al.*[19]. The present work provides another possibility that ubiquinone may act as a switch to channel electrons through the main phosphorylating pathway in the reduced form and through the shunt by the oxidized form which may result in the generation of extra heat.

The specificity studies of the diverse types of activators were aimed to probe into the nature of the effectors producing the physiological activation. The various properties were used as indices. The "hypobaric activation" was stable to washing with sucrose medium and only partially reversed on preincubation with succinate followed by washing and was also negative in the NT reduction. Essentially similar was the "hypoxic activation" except that the activation was completely reversed on preincubation with succinate followed by washing. The pairs that are sharing these properties are hypobaria–ubiquinol and hypoxia–malonate. While this is merely suggestive, compounds of similar nature or their derivatives, *e.g.* malonyl-CoA, may be the true activators. Also interesting to note is the difference in the response to the two stress conditions while both have an underlying lowered O_2 tension. Further work only can reveal the interrelationship of the stress, effector and regulation.

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REFERENCES

1 Kearney, E. B., Singer, T. P. and Zastrow, N. (1955) *Arch. Biochem. Biophys.* 55, 579
2 Kearney, E. B. (1957) *J. Biol. Chem.* 229, 363
3 Aithal, H. N. and Ramasarma, T. (1969) *Biochem. J.* 115, 77
4 Susheela, L. and Ramasarma, T. (1971) *Biochim. Biophys. Acta* 242, 532
5 Gutman, M., Kearney, E. B. and Singer, T. P. (1971) *Biochemistry* 10, 2726
6 Gutman, M., Kearney, E. B. and Singer, T. P. (1971) *Biochemistry* 10, 4763
7 Susheela, L. and Ramasarma, T. (1972) *Biochem. Biophys. Res. Commun.* 46, 2087
8 Aithal, H. N. and Ramasarma, T. (1971) *Biochem. J.* 123, 677
9 Ramasarma, T. and Lester, R. L. (1960) *J. Biol. Chem.* 235, 3309
10 Ramasarma, T. (1965) *Proc. Symp. High Altitude, Poona*, Publ. Indian Armed Forces Medical Services, p. 82
11 Arrigoni, O. and Singer, T. P. (1962) *Nature* 193, 1256
12 Slater, T. F. (1963) *Biochim. Biophys. Acta* 77, 365

13 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) *J. Biol. Chem.* 177, 751
14 Kimura, T., Hauber, J. and Singer, T. P. (1967) *J. Biol. Chem.* 242, 4987
15 Gregolin, C. and Scalella,P. 1965) *Biochim. Biophys. Acta* 99, 185
16 Tsou, C. L. (1951) *Biochem. J.* 49, 512
17 Lester, R. L. and Smith, A. L. (1961) *Biochim. Biophys. Acta* 47, 475
18 Albracht, S. P., Van Heerikhuizen, H. and Slater, E. C. (1972) *Biochim. Biophys. Acta* 256, 1
19 Singer, T. P., Gutman, M. and Kearney, E. B. (1971) *FEBS Lett.* 17, 11