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Activation of Liver Succinate Dehydrogenase in Rats Exposed to Hypobaric Conditions

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1. On brief exposure of rats to hypobaric conditions, the activity of hepatic mitochondrial succinate dehydrogenase was raised from the basal state to a 'partially activated state'. This was further raised to 'fully activated state' by preincubation of mitochondria with succinate, as was the activity in mitochondria from normal rats. 2. On washing mitochondria with the homogenizing sucrose medium the activity excess obtained on preincubation with succinate was lost in mitochondria from both normal and treated rats. 3. The enzyme in the 'partially activated state' from animals exposed to hypobaric conditions was stable to the washing procedure but was labilized and reverted to a low basal state of activity on freezing and thawing of the isolated mitochondria. 4. The results suggest that activation of succinate dehydrogenase under hypobaric conditions represents a conformational change leading to a stable, partially activated, form of the enzyme system: this is the first evidence of physiological modulation of this rate-limiting step in the control of the rate of oxidation of succinate.

Mammalian succinate dehydrogenase was activated severalfold on preincubation with succinate or other related substances capable of combining at the active centre (Kearney, Singer & Zastrow, 1955; Kearney, 1957), and to a smaller extent on exposure to elevated temperature (Thorn, 1962). This activation was observed in mitochondria prepared from a variety of animal tissues and also in solubilized preparations of the enzyme from bovine heart mitochondria (Kimura, Hauber & Singer, 1963). Extensive studies by Kimura, Hauber & Singer (1963, 1967) demonstrated that the activation was reversed on removal of the bound activating agent either by exclusion on Sephadex gel or by repeated washing. In view of the accompanying spectral shifts (Kearney, 1957; Dervartanian & Veeger, 1964) the activation was considered to be due to a change in the conformation of the protein, transforming it from a basal form, low in activity, into an 'activated state'. [The terminology used in the present paper is as follows: 'basal state', activity in mitochondria as isolated from normal animals; 'low-pressure-activated state' or 'partially activated state', activity in mitochondria from animals exposed to low atmospheric pressure; 'succinate-activated state' or 'fully activated state' or 'activated state', activity in mitochondria when preincubated with succinate as described by Kimura *et al.* (1967).] It is important to know at what state of activation the enzyme normally occurs *in vivo*. Modulation of activity of this enzyme would fit in with the well-known rate-limiting function of this step under various physiological conditions. The results presented in this paper show that, on brief exposure of intact rats to low atmospheric pressure and consequent hypoxia, succinate dehydrogenase activity in liver mitochondria was raised to a stable 'partially activated state'.

EXPERIMENTAL

Animals, exposure to low atmospheric pressure and preparation of liver mitochondria. Male albino rats (weighing 150–170g.) from the stock colony were exposed to an atmospheric pressure of 350 ± 5 mm. Hg, corresponding to an altitude of about 20000ft., in a decompression chamber fabricated in this laboratory (Ramasarma, 1965). Low pressure was obtained in the chamber in less than 3min. after the start of the evacuation and was maintained for 2, 4 or 6hr. At the end of the period atmospheric pressure was restored. The animals were then removed and killed by stunning and decapitation. After perfusion with cold 0·9% NaCl the liver was homogenized in 10vol. of cold 0·25M-sucrose medium [containing 0·01M-tris (pH 7·4) and 5mM-EDTA] in a Potter–Elvehjem homogenizer. Mitochondrial fractions were obtained by differential centrifugation by the procedure of Schneider & Hogeboom (1950), and were washed once with homogenizing medium and suspended in the medium. Protein was determined by the biuret method (Gornall, Bardawill & David, 1949). The mitochondrial suspension generally contained 10–15mg. of protein/ml.

Enzyme assays. Succinate dehydrogenase activity was determined spectrophotometrically with either DCI* alone or PMS–DCI as electron acceptor, by the methods of Green, Mii & Kohout (1955) and Arrigoni & Singer (1958) respectively. When DCI was used the assay mixture contained 50 μmoles of potassium phosphate buffer, pH 7·4, 1·5 mg. of bovine serum albumin, 6·0 μmoles of potassium cyanide (freshly neutralized), 0·04 μmole of DCI and 1–2 mg. of mitochondrial protein in a volume of 2·9 ml. The reaction was started by adding 50 μmoles of succinate (contained in 0·1 ml.) and the rate of reduction of DCI was followed by the decrease in E_{600} in a Beckman DB recording spectrophotometer. In the PMS–DCI method (Arrigoni & Singer, 1958) the reaction mixture contained 100 μmoles of potassium phosphate buffer, pH 7·6, 1·2 μmoles of potassium cyanide (freshly neutralized), 0·75 μmole of $CaCl_2$ and 1–2 mg. of mitochondrial protein in a volume of 2·7 ml. The reaction was started by the successive addition of 50 μmoles of succinate (in 0·1 ml.), 0·04 μmoles of DCI (in 0·1 ml.) and 0·1 ml. of 1% PMS solution, and the decrease in E_{600} was determined. The enzyme activity is expressed as units/mg. of protein, 1 unit being 1 μmole of DCI reduced/min.

Succinate oxidase and cytochrome oxidase activities were determined by measuring the oxygen uptake manometrically (Potter, 1957). The reaction mixture in the succinate oxidase system contained 100 μmoles of potassium phosphate buffer, pH 7·4, 0·04 μmole of cytochrome *c*, 1·2 μmoles of $CaCl_2$, 1·2 μmoles of $AlCl_3$, 150 μmoles of succinate and 1–2 mg. of mitochondrial protein in a volume of 3·0 ml. The oxygen uptake was measured for 30 min. after 5 min. of equilibration at 30°. The cytochrome oxidase system contained 100 μmoles of potassium phosphate buffer, pH 7·4, 0·25 μmole of cytochrome *c*, 1·2 μmoles of $AlCl_3$, 34 μmoles of ascorbate (neutralized) and 0·2–0·5 mg. of mitochondrial protein in a volume of 3·0 ml. The oxygen uptake at 30° was measured for 30 min. after an initial equilibration for 5 min. The enzyme activities are expressed as units/mg. of protein, 1 unit being the amount of oxygen utilized expressed as 1 μg.atom/min.

Preincubation of mitochondria with succinate. Mitochondria (1–2 mg.) were preincubated in potassium phosphate buffer with 50 μmoles of succinate in a volume of 1·3 ml. at 37° for 7 min. (Kimura *et al.* 1967). After the preincubation the enzyme activity was determined as described above.

Washing of the succinate-preincubated mitochondria. The activated preparation was diluted with 2 vol. of sucrose medium and sedimented at 8800*g* for 10 min. in a refrigerated Sorvall RC-2 centrifuge. The sediment obtained was again suspended in 2 vol. of the medium and recentrifuged. The pellet so obtained was suspended in 1 vol. of medium and was designated 'wash I'. The process was repeated once again to obtain the 'wash II' preparation. In these experiments generally about 50 mg. of mitochondrial protein was used.

RESULTS

Activation of succinate dehydrogenase during brief exposure to low atmospheric pressure. Progressive exposure of rats to low atmospheric pressure led to increased succinate oxidase activity of liver mitochondria, in confirmation of earlier observations (Criscuolo, Hale & Mefferd, 1958; Tappan, Reynafarge, Potter & Hurtado, 1957). This increase is apparently not due to overall enhancement of activity of the whole system, since only the activity of succinate dehydrogenase, the rate-limiting step, increased, not that of cytochrome oxidase (Fig. 1). The results in Table 1 show a small decrease in these enzyme activities in rats treated with cycloheximide, at the dose known to inhibit protein synthesis (Johnson, Hill, Alden & Ranhotra, 1966), but such treatment did not prevent the increase obtained during exposure of rats to low atmospheric pressure. In view of the increasing evidence that the mitochondrial enzyme proteins are synthesized at the ribosomal level (Gonzalez-Cadavid & Campbell, 1967; Roodyn, Suttie & Work, 1962), the use of cycloheximide is justified in these experiments. These, and other experiments described below, support the view that the increased succinate dehydrogenase activity was due, not to protein synthesis *de novo*, but to enhanced specific activity of the preformed enzyme.

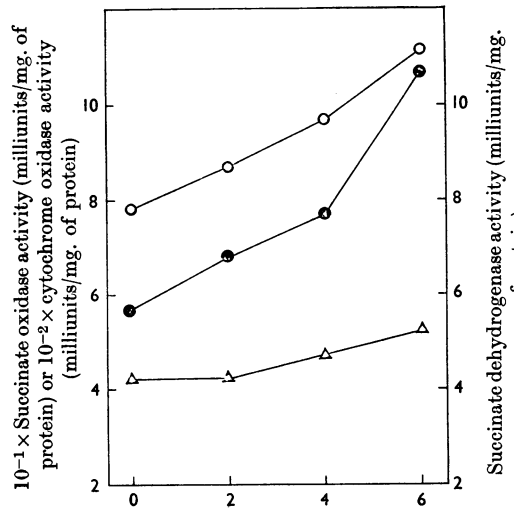


Fig. 1. Changes in the activities of the mitochondrial succinate oxidase system in livers of rats exposed to low atmospheric pressure. Rats were exposed to half-atmospheric pressure for 2, 4 or 6 hr. and the mitochondrial suspensions tested for succinate oxidase (○), succinate dehydrogenase (●) and cytochrome oxidase (△) as described in the Experimental section. The values represent the means of the samples independently processed from six rats in each set.

Effect of preincubation of mitochondria with succinate on succinate dehydrogenase. Extensive

* Abbreviations: DCI, 2,6-dichlorophenol-indophenol; PMS, *N*-methylphenazonium methylsulphate (phenazine methosulphate).

Table 1. *Effect of cycloheximide treatment of rats on the increased mitochondrial succinate dehydrogenase and succinate oxidase activities under hypobaric conditions*

Cycloheximide (250 μg./rat) was injected intraperitoneally into two groups of rats. After 30 min. one group was exposed to half-atmospheric pressure for 6 hr. in a decompression chamber. Two other groups were similarly treated but without being dosed with cycloheximide. All the animals were killed at the end of the experimental period (i.e. 6½ hr. after the injection of the drug). The activity of succinate dehydrogenase was determined with DCI as electron acceptor as described in the Experimental section. The values given are means ± S.D. with the numbers of animals used in parentheses. The increase obtained due to exposure to hypobaric conditions, with or without the drug, was statistically significant ($P < 0.01$), but the difference due to the drug treatment at each pressure was not ($P > 0.05$).

Activity	Treatment of rats	Enzyme activity (milliunits/mg. of protein) Normal pressure	Low pressure
Succinate dehydrogenase	None	6·1 ± 0·6 (12)	10·0 ± 2·3 (11)
	Cycloheximide	5·2 ± 1·8 (14)	10·3 ± 0·9 (6)
Succinate oxidase	None	70 ± 9 (12)	102 ± 16 (11)
	Cycloheximide	61 ± 19 (14)	94 ± 13 (6)

Table 2. *Effect of preincubation of mitochondria with succinate on the succinate dehydrogenase activity in livers of rats exposed to low atmospheric pressure*

The mitochondrial samples (1–2 mg.) were preincubated with 50 μmoles of succinate at 37° for 7 min. in a total volume of 1·3 ml. After the incubation the enzyme activity was determined with either DCI or PMS–DCI as the electron acceptor as described in the Experimental section. The values are the means of samples independently processed from two rats in each set. Low-pressure conditions were as described in Table 1.

Treatment of mitochondria	Assay method	Enzyme activity (milliunits/mg. of protein) Normal pressure	Low pressure
Unactivated	DCI	5·9	9·5
Preincubated with succinate	DCI	11·1	11·8
Unactivated	PMS–DCI	18·2	26·3
Preincubated with succinate	PMS–DCI	42·0	40·0

work by Kearney (1957), Kearney *et al.* (1955) and Kimura *et al.* (1963, 1967) showed that preincubation of mitochondria with succinate or other compounds capable of combining at the active site increased succinate dehydrogenase activity. It was decided to test whether activation after exposure to low pressure was of a similar nature. In the present set of experiments with rat liver mitochondria, incubation at 37° for 7 min. with succinate as described by Kimura *et al.* (1967), showed a two- to three-fold increase in activity of succinate dehydrogenase measured with either DCI or PMS–DCI as electron acceptor (Table 2). Incubation in 0·1 M-phosphate buffer, pH 7·4, alone without succinate also produced considerable activation. It was shown by Kearney (1957) that the activation phenomenon may be the same in all these cases. In all further experiments, therefore, preincubation in phosphate buffer in the presence of succinate at 37° for 7 min. was used as the standard procedure for obtaining full activation of the enzyme.

Mitochondria isolated from the livers of rats exposed to low atmospheric pressure were tested for activation by preincubation with succinate. The maximum activity reached in both the normal and the 'low-pressure-activated' mitochondrial samples was the same (Table 2). If the amount of enzyme protein had increased during exposure to low pressure, the activation by succinate should have been greater. These results therefore suggest an increased activity of the existing enzyme.

The effect of succinate concentration during assay on the activity of the enzyme was next studied. Both normal and 'low-pressure-activated' mitochondrial samples, either with or without prior incubation with succinate, gave the expected response to increasing substrate concentration (Fig. 12). Lineweaver–Burk plots gave the same K_m value for all the samples, indicating that kinetically the activation obtained either by preincubation of mitochondria with succinate or by exposure of rats to low atmospheric pressure is of 'V' type (Monod, Wyman & Changeux, 1965).

Regulatory effects by adenine nucleotides on the activation (or inhibition) by other metabolites of enzyme reactions are well known. A related typical example is the activation of acetyl-CoA carboxylase on preincubation with citrate (Martin & Vagelos, 1962) and the counteracting effect of ATP (Fang & Lowenstein, 1967). Addition of ATP or AMP at

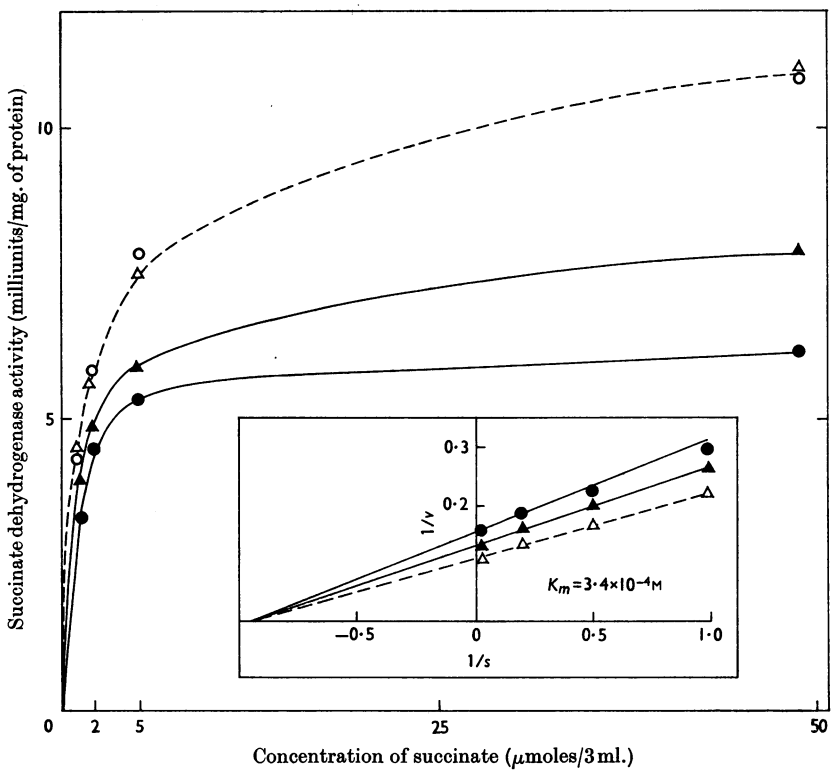


Fig. 2. Effect of succinate concentration on the activity of succinate dehydrogenase in the livers of rats exposed to low pressure. Various concentrations of succinate were added to two sets of tubes containing mitochondrial protein. One set was preincubated at 37° for 7 min. The succinate dehydrogenase activity in all the samples was tested with DCI as electron acceptor. The protein used for normal mitochondria both without preincubation (●) or with preincubation with succinate (○) was 2 mg., whereas that used for low-pressure-activated samples with preincubation (△) or without preincubation (▲) was 1·3 mg. The lowest succinate concentration used was sufficient for maximum activation during preincubation. Low-pressure conditions were as described in Table 1.

1 mM along with succinate during preincubation of mitochondria failed to show any effect.

Effect of washing of mitochondria on the 'activated form' of succinate dehydrogenase. Kimura *et al.* (1967) found that repeated washing of bovine heart mitochondria with homogenizing sucrose medium decreased the basal activity of succinate dehydrogenase in normal mitochondria. The activity could also be decreased to low values by Sephadex filtration of the purified soluble enzyme. This was considered to be due to removal of bound succinate or related metabolites. The conditions in the cell (succinate and other metabolites present; temperature 37°) are conducive to keeping the succinate dehydrogenase in an 'activated state'. Whether the washing procedure involved in the isolation of mitochondria from the cells decreases the activity was therefore tested. Samples of liver mitochondria from normal and 'low-pressure-exposed' rats were first activated by preincubation with succinate, and these activated preparations were then washed with homogenizing medium as described in the Experimental section. One washing of the 'succinate-activated' mitochondria caused the activity to revert to the initial value of the original preparation of isolated mitochondria. The activity could again be raised to the 'activated state' by preincubation with succinate at each stage of the washing procedure. The results with the 'low-pressure-activated' samples pretreated with succinate showed a similar response, with the activity reverting to the 'partially activated state' (Table 3). These results indicate that the activation induced by exposure to low pressure is distinct from that obtained on preincubation with succinate and probably represents a more stabilized state.

Effect of storage and freezing and thawing of mitochondria on the 'low-pressure-activated' succinate dehydrogenase. Since the enhanced activity might be due to structural alteration of the enzyme to an

Table 3. *Effect of washing on the activated mitochondrial succinate dehydrogenase activity in livers of rats exposed to low atmospheric pressure*

Mitochondria were preincubated with succinate and washed with the homogenizing medium by diluting with it and washing as described in the Experimental section. The values are the means of samples independently processed from two rats in each set. Low-pressure conditions were as described in Table 1.

	Enzyme activity (milliunits/mg. of protein)			
	Normal pressure		Low pressure	
Treatment of mitochondria	Unactivated	'Succinate-activated' in assay	Unactivated	'Succinate-activated' in assay
Initial state	17·0	41·0	26·0	40·0
Preincubated with succinate and washed once	18·0	39·6	24·2	40·5
Preincubated with succinate and washed twice	18·3	40·5	24·4	40·2

Table 4. *Effect of storage and repeated freezing and thawing of mitochondria on succinate dehydrogenase activity*

Mitochondria prepared from livers of rats at normal pressure or from rats maintained for 6 hr. at half-atmospheric pressure (see Table 1) were stored frozen in the sucrose medium at −20° for 30 days. One sample was thawed six times at regular intervals during the period and refrozen. The values are the means of samples independently processed from two rats in each set.

		Enzyme activity (milliunits/mg. of protein)	
Treatment of mitochondria	Assay method	Normal pressure	Low pressure
Initial state	DCI	5·9	9·6
Stored for 30 days	DCI	6·1	7·4
Stored for 30 days with six thawings	DCI	5·1	4·3
Initial	PMS–DCI	20·9	29·1
Stored for 30 days	PMS–DCI	21·0	23·0
Stored for 30 days with six thawings	PMS–DCI	16·8	10·6

activated conformation, it was decided to test whether it was reversed during storage and freezing and thawing of mitochondria. The results in Table 4 show that succinate dehydrogenase activity in normal mitochondria was stable during storage for 30 days in the frozen condition, but decreased with repeated thawings during the period. Under similar conditions the 'low-pressure-activated' succinate dehydrogenase activity decreased on storage and fell to a lower value with repeated thawings. These results show that the 'activated state' can be labilized during storage and freezing and thawing of mitochondria.

Protection of 'activated form' of succinate dehydrogenase by succinate during freezing and thawing. It is known that addition of a small concentration of succinate to the homogenizing medium during the preparation of mitochondria can protect and ensure higher activity of succinate oxidase. It was decided to test whether succinate could protect the 'activated form' of succinate dehydrogenase during storage and repeated freezing and thawing of mitochondria. Samples of mitochondria were stored before and after activation (incubation for 7 min. at 37°) in the presence of succinate in the frozen state at −20° and thawed at 5, 10 and 20 days and refrozen. The activity of samples was assayed at each stage. The results in Table 5 show that succinate dehydrogenase activity in normal mitochondria decreased to a small extent under these conditions and that the presence of succinate protected the activity. The 'low-pressure-activated' samples, however, could be protected only partly by succinate from loss of activity during freezing and thawing. 'Fully activated' forms obtained by preincubation with succinate were completely protected in the presence of succinate under these conditions. These preparations, which were 'succinate-activated' before storage, showed the same high activity after storage without further preincubation procedure.

DISCUSSION

The specific increase in succinate dehydrogenase in the liver of rats exposed to hypobaric conditions can be considered to be of physiological significance to the animal. Under such conditions the available oxygen in the tissues would be decreased. In acute exposure to such conditions the enzyme system might have to be regulated to provide minimal

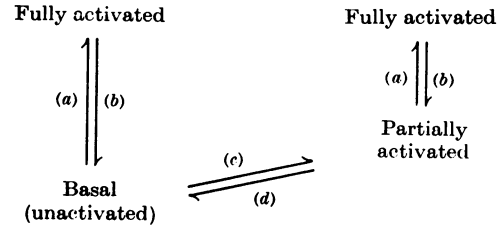
Table 5. *Protection by succinate of the activated succinate dehydrogenase during freezing and thawing and storage*

Mitochondrial samples were stored in the sucrose medium. Succinate (50 μmoles/ml. of medium) was added to two samples, one of which was incubated for 7 min. at 37°. All the samples were stored frozen at −20°. The tubes were thawed at 5, 10 and 20 days and portions assayed for succinate dehydrogenase. The values are means of samples independently processed from two rats in each set. Low-pressure conditions were as described in Table 1.

Storage at −20°		Treatment of mitochondria before storage		Enzyme activity (milliunits/mg. of protein)			
				Normal pressure		Low pressure	
Days	Thawings	Addition	Preincubation at 37° for 7 min.	Basal	'Succinate-activated' in assay	Basal	'Succinate-activated' in assay
Initial		—	−	18·0	40·5	25·3	39·0
5	1	—	−	17·3	39·1	16·0	37·3
		Succinate	−	18·0	40·4	20·7	39·1
		Succinate	+	39·9	—	41·0	—
10	2	—	−	17·0	39·4	15·9	37·7
		Succinate	−	18·1	40·8	19·5	39·1
		Succinate	+	39·8	—	40·6	—
20	3	—	−	15·8	37·6	15·2	37·6
		Succinate	−	17·6	41·3	18·2	38·6
		Succinate	+	38·3	—	39·2	—

energy requirements. In this context the modulation of the rate-limiting succinate dehydrogenase to increase the overall rate of oxidation of succinate would become meaningful 'to take advantage of the mass action effect' as suggested for such altered systems by Tappan *et al.* (1957). It is noteworthy that under conditions of oxygen excess succinate dehydrogenase activity was reported to decrease in *Astasia longa* (Begin-Heick & Blum, 1967). These effects could be explained in terms of the concept propounded by Racker (1965) that the influence of an external agent will be on the pacemaker enzyme 'with the capacity close to the rate-limiting factor of the pathway', rather than an enzyme present in large excess. Cytochrome oxidase is at sufficiently high concentration not to be rate-limiting. The limiting enzyme in the sequence of electron transport in this system is known to be succinate dehydrogenase, and this is the obvious site for a change to increase the overall rate despite lower oxygen concentration.

The remarkable feature underlying the observed effect is that physical exposure of the intact animal for a short period to low atmospheric pressure elicited what is apparently a structural change of an enzyme protein in liver mitochondria. The nature of this 'low-pressure activation' of succinate dehydrogenase deserves some comment. Since this 'partially activated state' was raised to the same 'fully activated state' as in normal mitochondria by preincubation with succinate, it is logical to compare the two mechanisms. There are at least two



Scheme 1. (*a*) Preincubation of the mitochondria with succinate; (*b*) washing of the mitochondria with homogenizing medium; (*c*) exposure of the whole animals to low pressure; (*d*) freezing and thawing of the mitochondria.

distinguishing features. First, activation obtained by succinate was reversed to the 'basal state' after one washing with sucrose medium in both types of preparations. Secondly, freezing and thawing of mitochondria deactivated the 'low-pressure-activated' samples even in the presence of succinate, whereas activation obtained by preincubation with succinate was completely stable on subsequent storage in the presence of succinate. It is not obvious whether the activation in 'low-pressure exposure' was caused by succinate or some related substance, which may be normally firmly bound but labilized during freezing and thawing. However, significantly these changes finally lead to the same maximal activity of the protein when the mitochondria are preincubated with succinate, suggesting that these may form part of the

alterations, albeit by different mechanisms, of the same active site. These results are represented in Scheme 1.

Observing the phenomenon of succinate activation, Thorn (1962) and Kimura *et al.* (1967) emphasized the importance of this activation and the need for its recognition in the correct measurement of succinate oxidation. It is apparent from our results that the basal activity would better represent the physiological activity of the enzyme system. Modulation of the activity of succinate dehydrogenase might control the oxidation rates of succinate. The present work supplies the first evidence of such a phenomenon occurring in intact animals.

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REFERENCES

Arrigoni, O. & Singer, T. P. (1958). *J. biol. Chem.* **234**, 666.
Begin-Heick, N. & Blum, J. J. (1967). *Biochem. J.* **105**, 813.
Criscuolo, D., Hale, H. B. & Mefferd, R. B. (1958). *J. appl. Physiol.* **13**, 353.
Dervartanian, D. V. & Veeger, C. (1964). *Biochim. biophys. Acta*, **92**, 233.
Fang, M. & Lowenstein, J. M. (1967). *Biochem. J.* **105**, 803.
Gonzalez-Cadavid, N. F. & Campbell, P. N. (1967). *Biochem. J.* **105**, 443.
Gornall, A. G., Bardawill, G. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Green, D. E., Mii, S. & Kohout, D. M. (1955). *J. biol. Chem.* **217**, 651.
Johnson, B. C., Hill, R. B., Alden, R. & Ranhotra, G. S. (1966). *Life Sci.* **5**, 385.
Kearney, E. B. (1957). *J. biol. Chem.* **229**, 363.
Kearney, E. B., Singer, T. P. & Zastrow, N. (1955). *Arch. Biochem. Biophys.* **55**, 579.
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.
Martin, D. B. & Vagelos, P. R. (1962). *J. biol. Chem.* **237**, 1787.
Monod, J., Wyman, J. & Changeux, J. P. (1965). *J. molec. Biol.* **12**, 88.
Potter, V. R. (1957). In *Manometric Techniques*, p. 170. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
Racker, E. (1965). *Mechanisms in Bioenergetics*, p. 198. New York: Academic Press Inc.
Ramasarma, T. (1965). *Proc. Indian Armed Forces, Medical Services Symp. High Altitude, Poona*, p. 82.
Roodyn, D. P., Suttie, J. W. & Work, T. S. (1962). *Biochem. J.* **83**, 29.
Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
Tappan, D. V., Reynafarge, B., Potter, V. R. & Hurtado, A. (1957). *Amer. J. Physiol.* **190**, 93.
Thorn, M. B. (1962). *Biochem. J.* **85**, 116.