3. Cellular organic radioiodine has been demonstrated in thyroids of rats pretreated with iodide, and labelled with ^{131}I for 10 and 15 min.

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The Kinetic Behaviour of Enzymes in Organized Systems

MITOCHONDRIAL SUCCINATE OXIDASE AND FUMARASE

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Effects of organization on the apparent kinetic parameters of enzymes give insight into the nature of the structural factors that may influence metabolic control. If two or more enzymes affected by a common substrate, inhibitor or activator are examined from this point of view, the relation between the composition of the external and internal medium of intact organized units, such as mitochondria, can also be elucidated. The present paper describes studies which illustrate two types of effects of organization on the kinetic behaviour of enzymes: control within closely linked reaction sequences and permeability barriers. These investigations are a continuation of those of Jones & Gutfreund (1963) on the control of succinate oxidation by the rate of electron transport and by competitive inhibition.

Previous studies (Chappell, 1962; Jones & Gutfreund, 1963) have shown that in several different types of mitochondria the rate of oxidation of succinate during closely coupled phos-

phorylation of ADP to ATP is limited by some step in the energy-conservation mechanism and not by the potential rate of the hydrogen and electron transfer from succinate to oxygen. Dinitrophenol or Ca^{2+} ions, which prevent the conservation of energy during electron transport, stimulate a considerable increase in the rate of oxidation of succinate. Studies with the pH-recording technique used by Gutfreund & Hammond (1963) to follow dinitrophenol-stimulated mitochondrial adenosine triphosphatase showed that stimulation by Ca^{2+} ions doubled the adenosine-triphosphatase activity compared with that with dinitrophenol (H. Gutfreund, unpublished work). The simplest kinetic scheme required for the analysis of our data can be represented as shown in Scheme 1, where E and E' are the oxidized and reduced succinate dehydrogenase respectively, S is succinate and F is fumarate. The step characterized by k_0 , the oxidation of reduced flavoprotein by the respiratory chain, involves the whole complex

of reactions affected by the process of energy conservation or uncoupling. The true Michaelis constant is defined by:

$$K_m = (k_{-1} + k_2)/k_1$$

and characterizes the steady-state concentration of the first complex formed between succinate and the enzyme. The different apparent Michaelis constants obtained from measurements under a variety of conditions are defined as:

$$K'_m = [(k_{-1} + k_2)/k_1] [k_0/(k_2 + k_0)].$$

This expression takes account of only two enzyme intermediates in the above scheme for succinate oxidation. This is, of course, an oversimplification, but it involves a sufficient number of constants to describe the observations made during the present investigation. This situation and the expression for K'_m is formally identical with the kinetic scheme for two enzyme-substrate intermediates that was developed by Gutfreund & Sturtevant (1956). If k_0 is much greater than k_2 the expressions reduce to the simple one-intermediate Michaelis-Menten scheme, whereas, if k_0 is of the same order of magnitude as or smaller than k_2 , then changes in k_0 have the mathematical form of uncompetitive inhibition (Segal, 1959). Changes in the rate of succinate oxidation by increasing k_0 cause a proportional rise in K'_m . Close coupling of succinate oxidation to rate-determining oxidative phosphorylation gives an apparent K'_m considerably smaller than that obtained for the isolated enzyme.

The second effect of organization on kinetic behaviour is illustrated by our studies of substrate and inhibitor availabilities. The consequences of a permeability barrier to an externally added substrate can be twofold. First, in the simple case of a diffusion barrier, the kinetic form of competitive inhibition would be observed: an increase in the apparent Michaelis constant and no change in the maximum velocity. If, however, there is some mechanism of substrate penetration through a limited number of active sites, a decrease in maximum velocity as well as an increase in the Michaelis constant can be observed.

Scheme 1.

METHODS AND RESULTS

All the experiments were carried out with guinea-pig-liver mitochondria which were prepared and used in the media described by Jones & Gutfreund (1963). The method of recording oxygen uptake with a Clark oxygen electrode was as described by Jones & Gutfreund (1962) except that the curvilinear recorder was replaced by a potentiometric recorder for more accurate evaluation of rates. The fumarase reaction was studied by analysis of the rate of appearance of malate (Hummel, 1949) in the presence of 3 mM-Amytal. All rate measurements were carried out at 25°.

Effect of calcium ions on succinate oxidation. The Ca^{2+} ion-stimulated swelling of mitochondria and uncoupling of oxidative phosphorylation have been reviewed by Lehninger (1962) (see also Hunter & Ford, 1955; Slater, 1957). With guinea-pig-liver mitochondria we observed that the initial rate of succinate oxidation on stimulation with calcium chloride (3 mM) was always between 3 and 4 times that observed during oxidative phosphorylation. Fig. 1 shows a number of features of Ca^{2+} ion-stimulated succinate oxidation: (1) the progress of oxygen uptake in the absence of Amytal is bi-phasic: (2) the initial rate is dependent on succinate

Fig. 1. Records of oxygen uptake during the oxidation of succinate by guinea-pig-liver mitochondria (approx. 3 mg. of protein/ml.) with other additions as indicated. The broken line represents the record of an experiment with Amytal (3 mM) and succinate (17 mM) added before the addition of mitochondria. The additions were made at the times indicated as follows: A, succinate (17 mM), or A', succinate (3.3 mM); B, mitochondria; C, CaCl_2 (3 mM); D, dinitrophenol (70 μM). The rates of oxygen uptake at X and Y were 0.09 and 0.18 $\mu\text{g. atom of O/min./mg. of protein}$ respectively.

concentration even when this is greater than 3 mM; (3) at the highest succinate concentration the rate of Ca^{2+} ion-stimulated oxidation is nearly twice that of dinitrophenol-stimulated respiration, the latter being independent of succinate concentration above 3 mM (Jones & Gutfreund, 1963). Fig. 2 shows that the stimulation by Ca^{2+} ions of NADH oxidation by mitochondria exhibits a lag phase. This lag phase corresponds to the more rapid phase of succinate oxidation. When glutamate is used as a substrate instead of succinate, one observes rapid initial respiration which decreases to zero rate of respiration during the period required to stimulate optimum oxidation of NADH.

The apparent K'_m for succinate oxidation stimulated by Ca^{2+} ions is clearly larger than that obtained by Jones & Gutfreund (1963) for dinitrophenol- or ADP-stimulated succinate oxidation. A precise value is difficult to obtain because one does not know how much endogenous respiration contributes to the total oxygen uptake. Series of measurements of oxygen uptake in the presence of 0.5–1.7 mM-succinate indicate that half the optimum rate is obtained at 1.0–1.5 mM-succinate.

Effect of malonate on succinate oxidation. Thorn (1953) has shown that, though malonate behaves as a truly competitive inhibitor for succinate during the oxidation of this substrate, the kinetic constants both for inhibition and oxidation are complex and variable with different preparations. Since K_i and K'_m are interdependent, in this section we quote only the values for K'_m/K_i determined by the method of Thorn (1953) at high substrate concentrations. Measurements of inhibition were carried out over the ranges 17–66 mM-succinate and 0.33–3.3 mM-malonate. The values for K'_m/K_i calculated from the initial rate of succinate oxidation on stimulation of mitochondria with 3 mM-calcium chloride reached a maximum value of approximately 30, decreasing at low relative concentrations of the inhibitor. The effect of malonate on ADP-stimulated succinate oxidation had the following feature. When the $[\text{I}]/[\text{S}]$ ratio is increased from zero no inhibition is observed until the value for $[\text{I}]/[\text{S}]$ is greater than 0.05. When $[\text{I}]/[\text{S}]$ equals 0.05 the rate of succinate oxidation stimulated by calcium chloride is decreased to the rate of ADP-stimulated succinate oxidation, the latter not being inhibited by this $[\text{I}]/[\text{S}]$ ratio. Fig. 3 illustrates this point. A plot of $V_{\text{max.}}/V_{\text{obs.}}$ against $[\text{I}]/[\text{S}]$ should, if a simple competitive inhibition mechanism is valid, extrapolate to $V_{\text{max.}}/V_{\text{obs.}}$ equals 1 when $[\text{I}]/[\text{S}]$ equals 0. The points for the ADP-stimulated succinate oxidation certainly come to $V_{\text{max.}}/V_{\text{obs.}}$ before $[\text{I}]/[\text{S}]$ equals 0, and this is probably also true for the points obtained from stimulation by Ca^{2+} ions. The kinetic implications of this are discussed below

together with the complex but related changes of K'_m and $V_{\text{max.}}$.

Effect of oxaloacetate on succinate oxidation. Jones & Gutfreund (1963) reported that oxaloacetate at relatively high concentration (1.7 mM-oxaloacetate; 3.3 mM-succinate) does not inhibit succinate oxidation linked to oxidative phosphorylation, whereas, on uncoupling of phosphorylation with dinitrophenol, inhibition occurs after a time lag. We have now compared the effect of a range of concentrations of oxaloacetate on succinate oxidation stimulated by ADP, dinitrophenol and calcium chloride. Experiments with calcium chloride-

Fig. 2. Records of oxygen uptake during the oxidation of 1 mM-NADH (solid line) and 3.3 mM-succinate (broken line) on the stimulation of guinea-pig-liver mitochondria (approx. 3 mg. of protein/ml.) by CaCl_2 (3 mM). The additions were made at the times indicated as follows: A, NADH (1 mM), or A', succinate (3.3 mM); B, mitochondria; C, CaCl_2 (3 mM).

Fig. 3. Plots of $V_{\text{max.}}/V_{\text{obs.}}$ against $[\text{I}]/[\text{S}]$ for the oxidation of succinate, S, by guinea-pig-liver mitochondria stimulated by ADP (3 mM) (O) and by CaCl_2 (3 mM) (●) in the presence of malonate, I. The points relating to ADP-stimulated oxidation are plotted on the upper scale for $[\text{I}]/[\text{S}]$ and the points relating to Ca^{2+} ion-stimulation are plotted on the lower scale.

stimulated mitochondria confirmed the very low value of K_i (less than $10\mu\text{M}$) reported by Pardee & Potter (1948). Fig. 4 (a) shows that, on the addition of oxaloacetate (final concentration 0.33 mM) to calcium chloride-stimulated mitochondria in the presence of 3.3 mM succinate, there is immediate complete inhibition of respiration, whereas (Fig. 4b) the stimulation of succinate oxidation with calcium chloride in the presence of succinate (3.3 mM) and oxaloacetate (0.33 mM) causes initial rapid respiration and complete inhibition after a lag phase similar to that reported above for other calcium chloride-stimulated respiration phenomena. Such low concentrations of oxaloacetate inhibit dinitrophenol-stimulated succinate oxidation only after a delay of approx. 1 min. The ADP-stimulated oxidation was not affected by additions of oxaloacetate up to a final concentration of 2 mM .

Effect of fumarate on succinate oxidation. Fumarate is known to behave as a competitive inhibitor for succinate oxidation. Singer, Kearney & Massey (1957) give a range of K_i of $1\text{--}1.9\text{ mM}$ for the inhibition of purified succinate dehydrogenase. Fig. 5 (a) shows that Ca^{2+} ion-stimulated succinate oxidation is competitively inhibited by fumarate. A series of such measurements showed that K_i is approximately equal to K_m . Fig. 5 (b), on the other hand, indicates that mitochondria oxidizing succinate on stimulation with dinitrophenol are only very slowly affected by the addition of fumarate. Identical results were obtained for the effect of fumarate on ADP-stimulated succinate oxidation.

Studies on fumarase activity in mitochondria. The initial rate of malate production on the addition of fumarate to mitochondria was studied over a range of fumarate concentrations in the presence and absence of 3 mM calcium chloride. Amytal (3 mM) was present to prevent the oxidation of malate produced. The results obtained were more variable than would be acceptable for data from purified enzyme preparations. This variation is likely to be due to the many factors involved in keeping mitochondria in prime condition. The fumarase activity of five preparations of mitochondria without Ca^{2+} ions was $0.075 \pm 0.025\mu\text{mole}$ of malate/min./mg. of mitochondrial protein. In the presence of Ca^{2+} ions the activity was $0.75 \pm 0.25\mu\text{mole/min.}$ The ratio of the activity of Ca^{2+} ion-stimulated to that of unstimulated mitochondria was always greater than 7. The apparent Michaelis constant for fumarase activity of mitochondria without Ca^{2+} ions was $4.5 \pm 2.5\text{ mM}$, whereas for the activity of mitochondria in the presence of Ca^{2+} ions no change in rate was observed on decreasing the fumarate concentration to 0.5 mM . This indicates that the apparent Michaelis

constant in the absence of Ca^{2+} ions was at least 10 times that in the presence of Ca^{2+} ions. Analogous results were obtained when intact mitochondria were compared with mitochondria frozen

Fig. 4. Records of oxygen uptake during the oxidation of succinate (3.3 mM) by guinea-pig-liver mitochondria (approx. 3 mg. of protein/ml.) on stimulation with 3 mM CaCl_2 : (a) on the addition of oxaloacetate (final concn. 0.33 mM) 75 sec. after treatment with CaCl_2 ; (b) on the addition of oxaloacetate (final concn. 0.33 mM) before stimulation of respiration with CaCl_2 . The additions were made at the times indicated as follows: A, succinate (3.3 mM); B, mitochondria; C, CaCl_2 (3 mM); D, oxaloacetate (0.33 mM).

Fig. 5. Records of oxygen uptake during the oxidation of succinate (3 mM) by guinea-pig-liver mitochondria in the presence of Amytal (3 mM) with additions of fumarate and succinate as indicated after the addition of: (a) CaCl_2 (3 mM); (b) dinitrophenol ($70\mu\text{M}$). In experiment (a) Amytal and succinate were present before the record started, and in experiment (b) Amytal, succinate and mitochondria were present before the record started. The other additions were made at the times indicated as follows: A, mitochondria; B, either (a) CaCl_2 (3 mM) or (b) dinitrophenol ($70\mu\text{M}$); C, fumarate (16 mM); D, succinate (20 mM).

at -20° and thawed: the activity of the frozen-and-thawed mitochondria corresponded to that of the calcium chloride-treated ones.

DISCUSSION

The analysis of the kinetics of a complex sequence of reactions catalysed by several enzymes, each of which forms a number of reaction intermediates, can yield useful information about the following points. When a substrate is utilized more rapidly than expected, alternative or additional enzymic pathways can be detected (Krebs & Bellamy, 1960; Borst & Slater, 1960; Jones & Gutfreund, 1961). Experimental conditions that change the apparent affinities of enzymes for their substrates can yield information about reaction intermediates and changes in rate-determining steps. Comparison of the behaviour of enzymes in isolation and in organized systems can give information about the effective concentrations of substrates and cofactors. In the present paper we have presented experimental results that allow us first to describe a linked system with variable rate-determining steps and secondly to obtain information about substrate availability to some mitochondrial enzymes.

We have made use of two effects of Ca^{2+} ions on mitochondrial enzyme systems. First, the addition of Ca^{2+} ions to a suspension of guinea-pig-liver mitochondria containing succinate causes a more rapid oxygen uptake than can be obtained by any other stimulation of succinate-oxidase activity of this system. Secondly, after a lag period of approximately 30 sec. from the addition of calcium chloride (3 mM), a structural change, which is interpreted throughout this discussion as a change in permeability, occurs. The first of these effects was utilized to study the kinetic effects of changes in the rate-determining step of the complex sequence of events during succinate oxidation, and the second effect allowed us to study the consequences of permeability barriers to some substrates of mitochondrial enzymes.

Slater & Bonner (1952) have pointed out that the Michaelis constant for succinate oxidation by pig-heart preparations is dependent on the concentration and efficiency of the electron-transport system and, when the rate of electron transport (k_0 in our scheme) approaches zero, $K_m = K_i = 30 \mu\text{M}$. We find with guinea-pig-liver mitochondria that, as the rate of electron transport is increased from ADP-stimulated to dinitrophenol-stimulated to Ca^{2+} -ion-stimulated, so the apparent Michaelis constant increases in proportion to k_0 (see also Jones & Gutfreund, 1963). This leads one to the conclusion that k_2 is very large compared with k_0 and k_{-1} .

Studies of malonate inhibition of succinate oxidation throw further light on the change of apparent K'_m with increase in electron transport. In Fig. 3 the points for malonate inhibition of ADP-stimulated succinate oxidation are plotted on a $[\text{I}]/[\text{S}]$ scale four times that for the points of the Ca^{2+} ion-stimulated oxidation. The line drawn corresponds to $K'_m/K_i = 6.5$ for ADP-stimulated oxidation and $K'_m/K_i = 26$ for Ca^{2+} ion-stimulated oxidation. We give this the following physical interpretation: the K_i for malonate is the same for all forms of stimulation of oxidation and the apparent K_m for Ca^{2+} ion-stimulated oxidation is four times that for ADP-stimulated oxidation. This is in agreement with our approximate K_m determinations and with the above conclusion that K_m is proportional to k_0 .

The true physical meaning of the constants proposed here and of the calculations proposed by Slater & Bonner (1952) will only be established from studies of the flavoprotein reactions of succinate dehydrogenase by such detailed methods as have been applied to xanthine oxidase (Gutfreund & Sturtevant, 1959) and lipoyl dehydrogenase (Massey, Gibson & Veege, 1960). It is likely that the differences in the rate of electron transport in mitochondria from different tissues and different species will result in differences of the ratios between the various steps of succinate oxidation. The type of experiments reported above should be applicable to obtain information about such ratios for other mitochondria.

The argument that externally added succinate and malonate are freely available to mitochondrial succinate dehydrogenase is based on this being consistent with all the available facts. The finding that succinate oxidation or malonate inhibition proceeds unchanged throughout the period of increasing Ca^{2+} ion-stimulated permeability, as long as Amytal is present to prevent the formation of oxaloacetate, also bears this out.

Our studies of the effect of oxaloacetate on succinate oxidation confirm (Jones & Gutfreund, 1963) that externally added oxaloacetate reaches succinate oxidase of intact mitochondria only very slowly. During ADP-stimulated succinate oxidation, oxaloacetate diffusing into mitochondria is utilized sufficiently rapidly to prevent any inhibition of oxidation, but during dinitrophenol-stimulated oxidation added oxaloacetate causes inhibition, after a lag phase, proportional to its external concentration. The Ca^{2+} ion-stimulated oxidation responds to the addition of oxaloacetate, as would be expected from its effect on the permeability of mitochondria, which allows free access of oxaloacetate to succinate dehydrogenase.

The biphasic behaviour of Ca^{2+} ion-stimulated succinate oxidation is presumably due to the

internal generation of oxaloacetate, which can be prevented by the addition of Amytal (Fig. 1). During the initial phase oxaloacetate accumulates, but, when the mitochondria become permeable and lose their NAD, oxaloacetate formation stops and a steady rate of oxidation is observed.

The lack of availability of added oxaloacetate to succinate dehydrogenase is in contrast with the ready availability of oxaloacetate generated in mitochondria by malate dehydrogenase and also to the ready availability of externally added oxaloacetate to enzymes for which it is a substrate: malate dehydrogenase (Chance & Gutfreund, 1963) and transaminase (Jones & Gutfreund, 1963).

The facts that fumarate added to calcium chloride-treated (permeable) mitochondria is freely available as a competitive inhibitor of succinate dehydrogenase and as a substrate for fumarase, though it is not freely available to either of these enzymes in intact mitochondria, clearly indicates a permeability barrier for this substrate. Quantitative conclusions about internal fumarate concentrations cannot yet be drawn from a comparison of the maximum rate of fumarase activity in intact and in calcium chloride-treated mitochondria. Though we know that permeability plays a major role in the differences of fumarase activity in intact and permeable mitochondria, it is also known that any difference in the internal ionic strength and phosphate concentration can also affect the activity of fumarase (Alberty, Massey & Frieden, 1954).

SUMMARY

1. Two different features of mitochondrial organization that affect the behaviour of enzymes have been studied with guinea-pig-liver mitochondria. First, in a closely coupled sequence of succinate oxidation, activation and inhibition of different steps have given information about the significance of the apparent K_m and V_{max} observed for the oxidation of succinate. Secondly, kinetic investigations with calcium chloride-treated (permeable) and with ADP- and dinitrophenol-treated (impermeable) mitochondria have given

information about the availability of added substrates to mitochondrial enzymes.

2. The Ca^{2+} ion-stimulated uncoupled succinate oxidation is approximately four times as fast as ADP-stimulated respiration, but its rate is probably still largely controlled by the respiratory chain. The apparent K_m for succinate is proportional to the rate of electron transport.

3. Added succinate and malonate are freely available to succinate dehydrogenase of intact mitochondria, but added oxaloacetate and fumarate are not. Added fumarate is also not freely available to intramitochondrial fumarase.

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