- Chappell, J. B. (1963). *J. biol. Chem.* **238**, 410.
- Chappell, J. B. & Greville, G. D. (1961). *Nature, Lond.*, **190**, 4775.
- Chappell, J. B. & Greville, G. D. (1963). *Symp. biochem. Soc.* **23**, 39.
- Ciotti, M. M. & Kaplan, N. O. (1957). In *Methods in Enzymology*, vol. 3, p. 890. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Cutolo, E. (1956). *Arch. Biochem. Biophys.* **64**, 242.
- Delbruck, A., Schimassek, H., Bartsch, K. & Bücher, T. (1959). *Biochem. Z.* **331**, 297.
- DeLuca, H. F., Gran, F. C., Reiser, S. & Steenbock, H. (1959). *J. biol. Chem.* **234**, 1912.
- Devlin, T. M. & Bedell, B. H. (1959). *Biochim. biophys. Acta*, **36**, 564.
- Dickens, F. (1959). In *A Conference on Enzymes and their Action, Wageningen*, p. 105. Zwolle: Tjeenk Willink.
- Dickman, S. R. (1961). In *The Enzymes*, vol. 5, p. 495. Ed. by Boyer, P. D., Lardy, H. A. & Myrback, K. New York: Academic Press Inc.
- Dickman, S. R. & Speyer, J. F. (1954). *J. biol. Chem.* **206**, 67.
- Ernster, L., Jalling, I., Löw, H. & Lindberg, O. (1955). *Exp. Cell Res. Suppl.* **3**, 124.
- Ernster, L. & Navazio, F. (1956). *Exp. Cell. Res.* **11**, 483.
- Ernster, L. & Navazio, F. (1957). *Biochim. biophys. Acta*, **26**, 408.
- Estabrook, R. W. (1957). *J. biol. Chem.* **230**, 735.
- Greville, G. D. & Chappell, J. B. (1959). *Biochim. biophys. Acta*, **33**, 267.
- Hager, L. P. & Gunsalus, I. C. (1953). *J. Amer. chem. Soc.* **75**, 5767.
- Hohorst, H. J., Kreutz, F. H. & Bücher, T. (1959). *Biochem. Z.* **332**, 18.
- Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
- Kolthoff, I. M. & Lingane, J. J. (1952). *Polarography*, p. 405. New York: Interscience Publishers Inc.
- Krebs, H. A. & Bellamy, D. (1960). *Biochem. J.* **75**, 523.
- Krebs, H. A., Eggleston, L. V. & D'Alessandro, A. (1961). *Biochem. J.* **79**, 537.
- Krebs, H. A. & Kornberg, H. L. (1957). *Ergebn. Physiol.* **49**, 212.
- Lardy, H. A., Johnson, D. & McMurray, W. C. (1958). *Arch. Biochem. Biophys.* **78**, 587.
- Lardy, H. A. & Wellman, H. (1952). *J. biol. Chem.* **195**, 215.
- Lehninger, A. L. (1951). *J. biol. Chem.* **190**, 345.
- Lehninger, A. L. & Greville, G. D. (1953). *Biochim. biophys. Acta*, **12**, 188.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Lotspeich, W. D. & Peters, R. A. (1951). *Biochem. J.* **49**, 704.
- Macarthur, C. G. (1916). *J. phys. Chem.* **20**, 495.
- McMurray, W. C., Maley, G. F. & Lardy, H. A. (1957). *J. biol. Chem.* **230**, 219.
- Peters, R. A., Sinclair, H. M. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 516.
- Purvis, J. L. (1958). *Biochim. biophys. Acta*, **30**, 440.
- Randall, M. & Failey, C. F. (1927). *Chem. Rev.* **4**, 271.
- Schneider, W. C. (1959). *Advanc. Enzymol.* **21**, 1.
- Seidell, A. (1919). *Solubilities of Organic and Inorganic Substances*, p. 470. New York: Van Nostrand Co.
- Siegel, J. M., Montgomery, G. A. & Bock, R. M. (1959). *Arch. Biochem. Biophys.* **82**, 288.
- Stein, A. M., Kaplan, N. O. & Ciotti, M. M. (1959). *J. biol. Chem.* **234**, 979.
- Stern, J. R. (1957). In *Methods in Enzymology*, vol. 3, p. 425. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Szent-Györgyi, A. V. (1939). *On Oxidation, Fermentation, Vitamins, Health and Diseases*, p. 50. Baltimore: Williams and Wilkins Co.
- Thorne, C. J. R. (1960). *Biochim. biophys. Acta*, **42**, 175.
- Vignais, P. V. & Vignais, P. M. (1961). *Biochim. biophys. Acta*, **47**, 515.
- Vogell, W., Bishai, F. R., Bücher, T., Klingenberg, M., Petter, D. & Zebe, E. (1959). *Biochem. Z.* **332**, 81.

Biochem. J. (1964) **90**, 237

The Effects of 2,4-Dinitrophenol on Mitochondrial Oxidations

By J. B. CHAPPELL

Department of Biochemistry, University of Cambridge, and the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa., U.S.A.*

(Received 29 May 1963)

Lardy & Wellman (1952) showed that the respiratory activity of carefully isolated liver mitochondria was low but that the rate of respiration could be increased several-fold by addition of ADP together with inorganic phosphate, or by other phosphate-acceptor systems, or by addition of various phenols and related compounds which act

by uncoupling oxidation and phosphorylation. Respiration may also be stimulated by structural damage to the mitochondria or by the addition of certain bivalent metal ions, e.g. Ca^{2+} , Mn^{2+} or Sr^{2+} , which are accumulated by the mitochondria (Brierley, Bachmann & Green, 1962; Chance, 1959; Chappell, Cohn & Greville, 1963; Chappell & Greville, 1963b; Lehninger, Rossi & Greenawalt, 1963a, b).

* Present address.

It has been claimed (see, for example, Chance, 1959; Slater & Hülsmann, 1959) that the maximal rate of respiration in the presence of uncoupling agents is equal, or nearly so, to the rate observed on stimulation of respiration by adenosine diphosphate together with inorganic phosphate. However, the investigations reported in this paper have revealed that this is in general not so. Of the substrates tested, only with glutamate were the rates of adenosine diphosphate-stimulated and 2,4-dinitrophenol-stimulated respiration equal. With liver mitochondria the rates of succinate and malate oxidation stimulated by dinitrophenol were 100–200 % greater than the rate with adenosine diphosphate, when the oxaloacetate produced was removed by transamination. Further, the rates of synthesis of adenosine triphosphate with both these last-named substrates was equal, indicating that in these cases the activity of the phosphorylation system was rate-limiting. In glutamate oxidation the rate-limiting reaction appears to be the conversion of α -oxoglutarate into succinate. Preliminary reports of this work have been published (Chappell, 1961*a*, *b*, 1962).

METHODS

The methods and reagents used in this investigation have been described by Chappell (1963, 1964). The preparation of pigeon-heart mitochondria, by using a proteinase (Chance & Hagihara, 1961), was kindly carried out by Miss Tsunekio Murakami. For the assay of oxidative activity, pigeon-heart mitochondria were suspended in 0.20M-mannitol, 0.05M-sucrose, 20 mM-triethanolamine hydrochloride, 5–10 mM-inorganic phosphate (P_i), 1 mM-ethylenediamine-tetra-acetate and the substrates indicated in the text and legends to Figures. The final pH was 7.2 and the temperature always used was 25°.

3-Fluoromalate and D- β -hydroxybutyrate were kind gifts from Dr P. W. Kent and Dr G. D. Greville respectively.

RESULTS

Before discussing the effects of uncoupling agents on mitochondrial oxidations it is desirable to define carefully the metabolic fate of the substrates used under the experimental conditions employed. Two simple one-step oxidations have been studied: the oxidation of succinate to fumarate and malate in the presence of 1.8 mM-Amytal (Borst & Slater, 1961), which inhibits the further oxidation of malate, and the oxidation of D- β -hydroxybutyrate to acetoacetate; acetoacetate is not further oxidized by liver mitochondria (Lehninger & Greville, 1953). The course of oxidation of isocitrate by mitochondria has been discussed by Chappell (1961*a*, 1964).

Oxidation of glutamate. Krebs & Bellamy (1960) have shown that alternative pathways of glutamate oxidation are possible in tissue preparations

and in mitochondrial preparations from different sources. These will be called the transaminase and dehydrogenase pathways. The former results in the conversion of glutamate into aspartate through the steps shown in reactions (1) and (2).

Reaction (1) is catalysed by the mitochondrial transaminase and reaction (2) is the result of the action of the α -oxoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase and fumarase. The dehydrogenase pathway involves the changes shown in reactions (3) and (4).

Reaction (3) is catalysed by the glutamate dehydrogenase and the steps in reaction (4) by the α -oxoglutarate dehydrogenase, succinate dehydrogenase and fumarase.

With liver mitochondria, glutamate oxidation appears to occur almost exclusively through the transaminase pathway (Borst & Slater, 1961) and analysis of the deproteinized reaction mixtures failed to reveal any increase in the amount of ammonia present or any significant malate production (see Table 1); if glutamate were oxidized by the dehydrogenase pathway ammonia would be produced. It was shown that with both rat-liver and pigeon-heart mitochondria malate was the end product of α -oxoglutarate oxidation. With rat-liver mitochondria oxidizing α -oxoglutarate in the presence of 100 μ M-dinitrophenol and 5 mM- P_i the ratio malate accumulating/oxygen consumed was 0.40–0.42 μ mole/ μ g.atom. If glutamate were oxidized by the dehydrogenase pathway malate accumulation would be expected, and this was not observed. However, in the presence of compounds that inhibit completely the conversion of α -oxoglutarate into oxaloacetate (reaction 2) the transaminase pathway is not possible. Thus 3-fluoromalate, a powerful competitive inhibitor of the mitochondrial malate dehydrogenase (Gal, 1960; Chappell, 1961*a*), allows the dehydrogenase pathway to proceed without contribution from the transaminase pathway. The effect of varying the concentration of 3-fluoromalate on the extent of inhibition of glutamate oxidation by rat-liver mitochondria is shown in Fig. 1(*a*). ADP-stimulated glutamate oxidation was approximately 50 % inhibited by 0.5 mM-3-fluoromalate and an eight-fold increase in concentration of the inhibitor produced no further effect, indicating that this

inhibitor probably had no secondary effects in this system. The degree of inhibition of glutamate oxidation was, however, dependent on the concentration of glutamate present (Fig. 1*b*). In the absence of fluoromalate the apparent Michaelis constant for glutamate was 0.6–0.8 mM, whereas in the presence of the inhibitor the corresponding constant was 5 mM. This latter value was independent of the concentration of inhibitor present over the range 0.5–4 mM-fluoromalate.

Experiments with liver mitochondria that had been lysed by addition of the detergent Triton X-100 and then suspended in the presence of 1 mM-NAD and 1 μ M-cytochrome *c*, revealed that fluoromalate did not inhibit glutamate oxidation under these conditions. α -Oxoglutarate was not oxidized by these disrupted mitochondria and the observed oxygen uptake must have been due to glutamate-dehydrogenase activity. Since the rate of oxygen consumption was 150 % greater than the rate with intact mitochondria oxidizing glutamate, it follows that the low rate of glutamate oxidation through the dehydrogenase pathway, with liver mitochondria, was not due to a low glutamate-dehydrogenase activity, but was due to some 'latency' phenomenon (see Chappell & Greville, 1961, 1963*a*). This is to be contrasted with the situation with heart mitochondria, where a deficiency of glutamate dehydrogenase appears to be responsible for the very low rate of oxidation through the dehydrogenase pathway (Borst & Slater, 1960).

From the results presented in Table 1 it is possible to assess the maximum rates at which the individual steps involved in glutamate oxidation occur. Thus, of the three oxidative steps involved

in the transaminase pathway, the rate of conversion of α -oxoglutarate into succinate was the slowest, and it would be expected that this would be the rate-limiting step. In fact the rate of oxygen uptake in the presence of glutamate was very nearly three times the rate at which α -oxoglutarate was converted into succinate (in the presence of malonate). This was the case when both ADP and dinitrophenol (at concentrations in excess of 20 μ M) were used to stimulate respiration, and with both rat-liver and pigeon-heart mitochondria. Since, in the presence of β -chlorovinylarsenious oxide, malate is converted into aspartate at rates of up to 0.22 μ mole/mg. of protein/min. with rat-liver mitochondria and 0.17 with pigeon-heart mitochondria, it follows that transaminase activity is not limiting in glutamate oxidation. The rate-limiting step in the dehydrogenase pathway would appear to be the rate at which glutamate is converted into α -oxoglutarate and ammonia. If it is assumed that, in the presence of fluoromalate, 3 atoms of oxygen are consumed for each molecule of glutamate metabolized and, in the presence of malonate, 2 atoms, then the rate at which intact liver mitochondria are able to catalyse the glutamate-dehydrogenase step may be calculated to correspond to an oxygen consumption of 0.017 μ g.-atom/mg. of protein/min. This is to be compared with the rate of 0.25 for lysed mitochondria oxidizing glutamate to α -oxoglutarate and ammonia in the presence of NAD and cytochrome *c*. Neither of these compounds affected the rate of oxidation of glutamate by untreated mitochondria.

Malate oxidation. In the presence of β -chlorovinylarsenious oxide, glutamate oxidation is

Table 1. *Effect of inhibitors on glutamate oxidation by rat-liver and pigeon-heart mitochondria*

Substrates were present at 10 mM concentration. Temp. 25°, pH 7.2.

Substrate	Inhibitor present	Rate of respiration (μ g.atom of O/mg. of protein/min.)			Remarks
		Rat liver		Pigeon heart: ADP-stimulated	
		ADP-stimulated	50 μ M-Dinitrophenol		
Glutamate	None	0.090–0.105	0.090–0.105	0.265–0.278	Aspartate only* detectable product
Glutamate	5 mM-Malonate	0.030–0.035	0.030–0.035	0.006–0.008	Dehydrogenase pathway
Glutamate	1 mM-Fluoromalate	0.048–0.052	0.048–0.052	0.009–0.010	Dehydrogenase pathway
α -Oxoglutarate	None	0.064–0.066	0.064–0.067	0.160–0.180	End product, malate†
α -Oxoglutarate	5 mM-Malonate	0.030–0.035	0.030–0.035	0.085–0.090	
Glutamate + malate	β -Chlorovinylarsenious oxide (1 μ g./2 ml.)	0.12–0.135	0.20–0.22	0.16–0.17	Respiration due to malate-dehydrogenase activity only
Succinate	1.8 mM-Amytal	0.16–0.20	0.28–0.32	0.20–0.22	End products, malate and fumarate (Fig. 3)

* Steady-state level of malate was less than 20 μ M.

† Simultaneous determination of the malate produced indicated that the ratio μ g.atom of O consumed/ μ mole of malate produced was 0.40–0.42.

almost completely inhibited (Chappell, 1961*a*), but glutamate is still able to act as a transaminating agent and under these conditions malate is rapidly oxidized. Also when P_i is omitted from the incubation medium, on addition of dinitrophenol neither glutamate nor α -oxoglutarate is oxidized to any significant extent. Cysteinesulphinic acid may be used to replace glutamate. This compound has not been used extensively in these investigations since the β -sulphinyldipyrivate, which arises as the product of transamination, decomposes to pyruvate and sulphite, and the latter compound is oxidized to sulphate by liver mitochondria (Singer & Kearney, 1956).

Fig. 1. Effect of 3-fluoromalate on ADP-stimulated glutamate oxidation by rat-liver mitochondria at 25° and pH 7.2. (a) Effect of fluoromalate concentration on the initial rate of glutamate oxidation. Concentration of P_i was 5 mM and 500 μM-ADP was added to stimulate respiration. (b) Effect of variation of glutamate concentration on the initial rate of respiration in the presence of 1 mM-fluoromalate (○) and in the absence of the inhibitor (×). Values were corrected for an endogenous rate of respiration of 0.008 μg. atom of O/mg. of protein/min.

Stimulation of respiration by dinitrophenol

At pH 7.2 and with rat-liver mitochondria suspended in the presence of 10 mM-glutamate and 10 mM-succinate, but with no P_i present (under these conditions added α -oxoglutarate is not oxidized at a rate significantly greater than the endogenous rate of respiration and glutamate serves as a transaminating agent), 20 μM-dinitrophenol produced the same rate of oxygen uptake as that observed with ADP and P_i together. A further increase in dinitrophenol concentration led to higher oxidative activity; at 100 μM-dinitrophenol the respiratory activity was 2.2 times as great as the ADP-stimulated rate (Fig. 2). In the presence of 1.8 mM-Amytal, the rate of oxygen uptake at lower dinitrophenol concentrations was little affected, but at higher concentrations Amytal inhibited; when ADP and P_i were used, Amytal had virtually no inhibitory action, leaving both the rate of oxidation and the P:O ratio unchanged. The effect of this barbiturate suggests that only at higher dinitrophenol concentrations was malate oxidation contributing to the oxygen uptake. This was confirmed in the experiment shown in Fig. 3, which was conducted under precisely the same

Fig. 2. Effect of variation of concentration of dinitrophenol on the rate of oxidation of 10 mM-succinate and 10 mM-glutamate in the presence (Δ) and absence (○) of 1.8 mM-Amytal. Temp. 25°, pH 7.2. No P_i was added. The rate of respiration obtained in the presence of 5 mM- P_i and 500 μM-ADP (broken line) is shown for comparison. Extent of inhibition by Amytal is also shown (●).

conditions as those prevailing in the experiment shown in Fig. 2. Under these conditions three main products of succinate oxidation, namely fumarate, malate and aspartate, are to be expected. There-

Fig. 3. Accumulation of malate in the incubation medium when (a) pigeon-heart mitochondria (O) and (b) rat-liver mitochondria (Δ) oxidized 10 mM-succinate in the presence of 10 mM-glutamate. Malate was assayed after the reaction had been stopped at the point at which the suspension became anaerobic, that is after the consumption of 0.48 $\mu\text{g. atom}$ of O/ml. in all cases. In the experiments with rat-liver mitochondria no P_i was added. With the pigeon-heart mitochondria 5 mM- P_i and 1 $\mu\text{g.}$ of β -chlorovinyl-arsenious oxide were present. Temp. 25°, pH 7.2. ●, Amounts of malate found when 500 μM -ADP and 5 mM- P_i were used to stimulate respiration; broken lines, amounts of malate produced in the presence of 1.8 mM-Amytal and 100 μM -dinitrophenol.

Fig. 4. Effect of concentration of dinitrophenol on the rate and linearity of the rate of succinate oxidation by rat-liver mitochondria. Conditions: 10 mM-succinate, no P_i , 25°, pH 7.2, 1.5 mg. of protein/ml. Initial rates of respiration (O) were maintained until the oxygen shown (Δ) had been consumed. At 20 μM -dinitrophenol the suspension became anaerobic before a decrease in rate occurred.

fore by determining the amount of malate formed and neglecting the contribution of fumarate, it may be deduced to what extent malate oxidation had occurred. In the presence of ADP and P_i , or at low dinitrophenol concentrations, amounts of malate accumulated in the medium equivalent to that observed in the presence of 1.8 mM-Amytal, conditions under which malate oxidation could not occur. It must be emphasized that glutamate was present in all these experiments. At higher dinitrophenol concentrations the yield of malate decreased, corresponding to the increased rate and the Amytal sensitivity of the oxygen uptake. Qualitative paper chromatography (Krebs & Bellamy, 1960) of portions of the incubation mixture revealed that aspartate was produced only in the presence of high dinitrophenol concentrations. In the presence of ADP and P_i , or at low dinitrophenol concentrations, no aspartate could be detected. The conclusions from these experiments are that with liver mitochondria (1) dinitrophenol concentrations less than 20 μM and (2) ADP in the presence of P_i permitted only succinate oxidation and not the further metabolism of the malate produced; with increasing dinitrophenol concentrations malate oxidation occurred.

In the absence of added glutamate the stimulation of succinate oxidation by dinitrophenol was quite different and, with liver mitochondria, at concentrations of dinitrophenol in excess of 20 μM non-linear rates of succinate oxidation were obtained; there was a burst of oxidative activity followed by marked inhibition (Fig. 4). This result is consistent with the findings described above, since it has been shown that at higher dinitrophenol concentrations malate oxidation occurred and this would result in oxaloacetate production (since no transaminating agent was present) and this in turn would lead to inhibition of succinate oxidation (Pardee & Potter, 1948). Consistent with this is the finding that the inhibition can be prevented in a variety of ways, all of which lead to the removal of oxaloacetate or prevent its production (Chappell, 1963).

Another aspect of the inhibitory action of dinitrophenol on succinate oxidation is that the effect of oxaloacetate itself probably permits further malate oxidation; that is, the process is 'auto-inhibitory'. Thus when 20 μM -dinitrophenol was used to stimulate succinate oxidation linear rates of oxygen consumption were obtained, but in the presence of 0.25 mM-malonate (Fig. 5) a progressive decrease in activity occurred; in the presence of Amytal and malonate a linear rate of oxidation was observed. This result suggests that because of the decreased activity of the succinate dehydrogenase in the presence of malonate, malate oxidation occurred and oxaloacetate production led to a

progressively decreasing rate of succinate oxidation. In the presence of isocitrate, which served to remove oxaloacetate, linear rates of oxidation were obtained. The inhibition of succinate dehydrogenase by malonate is rapid, whereas that produced by oxaloacetate is not (Chappell, 1961a).

The importance of the relative activity of the succinate dehydrogenase, and of the rate at which electron transport through the cytochrome system occurs, in determining the course of succinate oxidation is best demonstrated by considering the oxidation of succinate by pigeon-heart mitochondria. Addition of ADP to a suspension of pigeon-heart mitochondria in the presence of succinate and P_i led to an atypical course of oxidation (Fig. 6). Immediately after addition of ADP there was a burst of oxygen consumption of short

Fig. 5. Oxygen-electrode traces of the effect of $20\mu\text{M}$ -dinitrophenol (added at the second arrow on each curve) on the course of respiration with 10 mM-succinate as substrate under various conditions: (a) control experiment; (b) effect of adding 0.25 mM-malonate; (c) and (d) effects of adding 1.8 mM-Amytal and 5 mM-isocitrate respectively in the presence of succinate and malonate. Figures on the curves are the rates of oxygen uptake ($\mu\text{g. atom of O/mg. of protein/min.}$). Temp. 25° , pH 7.2. First arrow on each curve represents point of addition of mitochondria.

Fig. 6. ADP-stimulated oxidation of 10 mM-succinate by pigeon-heart mitochondria. Right-hand curve, response to ADP ($300\mu\text{M}$) with succinate only; left-hand curve, effect of adding 1.8 mM-Amytal at zero time. A and B represent points of addition of mitochondria and ADP respectively. Temp. 25° , pH 7.2.

duration followed by a decreased rate, which was then followed by a slow increase which coincided with a lowering of the ADP concentration, presumably, since addition of more ADP at this stage prevented the increase in rate. When the ADP had been depleted the rate characteristic of state 4 resulted. Further addition of ADP led to a repetition of the same behaviour. In the presence of Amytal a more usual pattern of oxidation was obtained, indicating that malate oxidation was involved when the barbiturate was not present. Confirmation of this was obtained from experiments of the kind shown in Fig. 3. Pigeon-heart mitochondria were allowed to oxidize succinate in the presence of glutamate, P_i and β -chlorovinyl-arsenious oxide (to prevent glutamate oxidation). In contrast with the findings with rat-liver mitochondria, with the particles from pigeon heart the amount of malate accumulated was low when ADP and P_i were used to stimulate respiration and corresponded to that obtained with high concentrations of dinitrophenol. Only at low concentrations of dinitrophenol were significant quantities of malate accumulated and under these conditions linear rates of dinitrophenol-stimulated oxidation were also obtained.

It is possible to reduce the rate of ADP-stimulated electron transport through the cytochrome system by decreasing the rate at which ATP synthesis occurs, by using small amounts of oligomycin (Lardy, Johnson & McMurray, 1958). In Fig. 7 the effects of increasing amounts of oligomycin on the rates of oxidation of a mixture of glutamate and succinate and of ATP synthesis (calculated from the P:O ratio and rate of respiration) are shown. It is significant that, as the amount of oligomycin was increased, the P:O ratio fell towards 2, indicating perhaps that at this stage succinate oxidation was predominating. Such titrations, when succinate was not present as a substrate (e.g. with malate), did not reveal a decrease in the P:O ratio. With the data from Fig. 7 it was possible to decrease the phosphorylation activity of pigeon-heart mitochondria to any extent required. When this was done, linear rates of succinate oxidation by pigeon-heart mitochondria could be obtained (Fig. 8). However, even in the presence of oligomycin addition of $100\mu\text{M}$ -dinitrophenol caused inhibition of succinate oxidation. Oligomycin does not decrease the rate of dinitrophenol-stimulated respiration (Lardy *et al.* 1958). Subsequent addition of glutamate restored oxidative activity, again indicating that oxaloacetate production was responsible for inhibition.

Taken together the experiments described above indicate that the relative activity of the succinate dehydrogenase and the rate at which electron transport occurs are of prime importance in deter-

mining the course of succinate oxidation. With rat-liver mitochondria in the presence of ADP and P_i , because of the relatively slow rate of ATP synthesis and consequently, because of coupling of electron transport, little malate oxidation occurred and linear rates of succinate oxidation were obtained. With pigeon-heart mitochondria, which have a more active system for ATP synthesis (see below), the relative activities of the succinate dehydrogenase and the phosphorylation system were such

that malate oxidation occurred and non-linear rates of succinate oxidation resulted, unless oxaloacetate was removed.

*Maximum rate of synthesis of
adenosine triphosphate*

It appears that, under certain conditions, the rate at which electron transport occurs in a phosphorylating system is limited by the rate at which the mitochondria are able to synthesize ATP. It may be further predicted that there should be, under given conditions, a characteristic maximum rate at which ATP can be synthesized. Obviously for those substrates which are poorly oxidized the rate of ATP synthesis must be less than maximal, but a combination of such substrates should again lead to maximal rates, other things being equal. In Table 2 are shown the results of experiments in which a single preparation of rat-liver mitochondria was used to determine the rates of oxygen uptake and of ATP synthesis in the presence of the substrates and combination of substrates shown and at four different temperatures. (1) At the temperature tested, the rates of synthesis of ATP by rat-liver mitochondria in the presence of (a) succinate, (b) succinate together with glutamate, (c) malate together with glutamate, (d) isocitrate together with malate and (e) D- β -hydroxybutyrate together with glutamate, were within experimental error the same. (2) At 38° the rate of ATP synthesis with D- β -hydroxybutyrate was the same as that with the substrates and combination of substrates mentioned. In all these cases, except with D- β -hydroxybutyrate, the addition of optimum amounts of dinitrophenol produced rates of electron transport in excess of those obtained with ADP and P_i , again indicating that ATP synthesis was rate-limiting. Fig. 9 shows the effect of dinitrophenol concentration on the rate of oxidation of a mixture of glutamate and malate at four temperatures. There was a marked temperature-dependence of the inhibitory effect of excess of dinitrophenol. This behaviour was not noted when glutamate together with succinate were used as substrates.

When glutamate also served as substrate the maximal rate of dinitrophenol-stimulated respiration was equal to that obtained with ADP and P_i . In this case, and for the reasons given above, it is thought that the rate at which α -oxoglutarate is converted into succinate controls the rate of glutamate oxidation.

The maximal rate of ATP synthesis determined with pigeon-heart mitochondria has not been thoroughly investigated, but the rate of synthesis of ATP with glutamate together with succinate, or with glutamate together with malate, was the same and gave values 0.95–1.20 μ moles of ATP/mg. of protein/min. at 25° (Chappell, 1963).

Fig. 7. Effect of oligomycin concentration on the rate of ADP-stimulated respiration (○), P:O ratio (●) and ATP synthesis (△) by pigeon-heart mitochondria suspended in the presence of 10 mM-glutamate, 10 mM-succinate and 5 mM- P_i . Temp. 25°, pH 7.2.

Fig. 8. ADP-stimulated succinate oxidation in the presence of oligomycin (0.6 μ g./2 ml.). Other conditions were as described in the legend to Fig. 6. Note that no glutamate was added.

pH-dependence of phosphorylation rate

In confirmation of the findings of Chance & Conrad (1959), a single broad optimum for the rate of phosphorylation of ADP by rat-liver mitochondria was obtained, the maximum lying at approximately pH 7.2. No evidence for multiple optima of the kind described by Slater & Hülsmann (1959) could be obtained.

The ability of dinitrophenol to produce greater rates of electron transport was even more marked at alkaline pH values greater than 7.2. Thus at pH 8.2 the rate of ADP-stimulated oxidation of malate together with glutamate was only 22 % of that at pH 7.2, but 200 μ M-dinitrophenol produced a rate of respiration that was 92 % of the maximal rate induced by dinitrophenol at pH 7.2. At pH 7.2 and at 25°, 50 μ M-dinitrophenol caused a rate of oxygen uptake which was 95 % greater than the ADP-stimulated rate of respiration; at pH 8.2 the rate with dinitrophenol was 800 % greater than the ADP-stimulated rate. A precise investigation of the pH-dependence of dinitrophenol-stimulated respiration is rendered difficult by the effect of pH on the ionization of dinitrophenol and by the inhibitory effects of dinitrophenol itself (see Fig. 9). The highest rate of respiration in the presence of dinitrophenol, at optimum concentration, was

observed at pH 7.5 with succinate, or malate together with glutamate as substrates, but the optimum was very broad. At pH values in excess of 8.5 the dinitrophenol response rapidly decreased.

At pH values more acid than 6.5, dinitrophenol, at all concentrations tested, produced an extremely small stimulation of respiration, and in the course of 1–2 min. inhibition occurred. This effect was reversible, since, when the suspension was adjusted to pH 7.2, the rates of respiration were the same as those that would have been obtained with the amounts of dinitrophenol added, had the mitochondria never been exposed to acid conditions.

Requirement for phosphate for dinitrophenol-stimulated respiration

In confirmation of the findings of Borst & Slater (1961), no requirement for added P_i could be shown for the dinitrophenol-stimulated oxidation by rat-liver mitochondria of substrates other than glutamate, α -oxoglutarate and isocitrate (Chappell, 1961*a*, 1964). This was the case even at the relatively high rates of electron transport induced by 100–200 μ M-dinitrophenol, under the conditions defined above, and in the presence of oligomycin. Since the latter compound almost completely inhibits the dinitrophenol-stimulated adenosine triphosphatase and the ATP- P_i -exchange reaction

Table 2. *Effect of temperature on the rate of synthesis of adenosine triphosphate by rat-liver mitochondria*

Rates of ATP synthesis were obtained from the rate of oxygen consumption in the presence of ADP and the P:O ratio determined by the method of Chance & Williams (1955). Concentrations of substrates: 10 mM-succinate, 10 mM-glutamate, 5 mM-D- β -hydroxybutyrate, 5 mM-L₆(+)-isocitrate and 1 mM-malate.

Substrate	Temp.	Rate of ATP synthesis (μ mole of ATP/mg. of protein/min.)			β -Hydroxy- butyrate
		Succinate	Glutamate	Malate	
Succinate	20°	0.21	—	—	—
	25	0.36	—	—	—
	30	0.56	—	—	—
	38	0.68	—	—	—
Glutamate	20	0.22	0.14	—	—
	25	0.36	0.29	—	—
	30	0.57	0.32	—	—
	38	0.69	0.48	—	—
Malate	20	—	0.20	0.03	—
	25	—	0.36	0.06	0.25
	30	—	0.55	0.08	—
	38	—	0.70	0.15	0.64
β -Hydroxybutyrate	20	—	—	—	0.14*
	25	—	0.34	—	0.25*
	30	—	—	—	0.37*
	38	—	0.65	—	0.65
Isocitrate	20	—	—	0.12	—
	25	—	—	0.25	0.35
	30	—	—	0.35	—
	38	—	—	0.52	0.67

* Obtained from initial rates.

(Lardy *et al.* 1958), the fact that this inhibitor did not affect the rate of respiration at any concentration of the phenol precludes the possibility that the dinitrophenol-elicited adenosine triphosphatase plays a significant role in dinitrophenol-stimulated respiration. Indeed these findings lend strong support to the view that dinitrophenol acts at a site before the entry of ADP and P_i into the phosphorylation process (Slater & Hülsmann, 1959; Borst & Slater, 1961)

However, in marked contrast with the findings with rat-liver mitochondria, P_i was required for maximal rates of dinitrophenol-stimulated oxidation of both succinate and malate (in the presence of glutamate and β -chlorovinylarsenious oxide) by pigeon-heart mitochondria. Thus before significant rates of dinitrophenol-stimulated oxidation could be demonstrated it was necessary to incubate the mitochondria with both substrate and P_i . This was the case when Amytal or a transaminating system was present and, under both these sets of condi-

tions, interference from the inhibitory effects of oxaloacetate would be expected to be minimal (see Azzone & Ernster, 1960). It may even be that the mechanism of stimulation of respiration by dinitrophenol in heart mitochondria is different or that P_i is required for the process of electron transport itself in this case (Bonner, 1954).

DISCUSSION

At the relatively low rates of electron transport obtained on addition of ADP and P_i to liver mitochondria or of low concentrations of dinitrophenol to either liver or pigeon-heart mitochondria, succinate was oxidized to fumarate and malate; this latter compound was not oxidized to any significant extent. Only when the concentration of dinitrophenol was increased, or with pigeon-heart mitochondria on addition of ADP (or alternatively when the activity of the succinate dehydrogenase was reduced by addition of malonate), was significant malate oxidation observed.

Perhaps the most important feature to be considered is the competition between the succinate dehydrogenase and malate dehydrogenase for oxidized cytochrome *b*, or some other common component at approximately this level in the electron-transport chain (Scheme 1). Solely on the basis of the relative activity of the two dehydrogenases (Table 1) there is no reason to believe that the succinate would be markedly more effective than the malate dehydrogenase in reducing cytochrome *b*. However, the succinate dehydrogenase is able to reduce cytochrome *b* without any requirement for ADP and P_i , or for intermediates liberated as a result of the action of dinitrophenol or other uncoupling agents; in a coupled system, before $NADH_2$ can reduce cytochrome *b* these components must be available. For convenience it may be assumed that some intermediate $X \sim I$ is synthesized from compound *X* and *I* as a result of electron transport (reaction A), that either ADP together with P_i , or dinitrophenol, leads to the

Fig. 9. Effect of concentration of dinitrophenol and temperature on the rate of oxidation of 10 mM-malate and 10 mM-glutamate by rat-liver mitochondria. No P_i was added. pH 7.2.

Scheme 1. Representation of the cytochrome chain and phosphorylation system in relation to succinate and malate oxidation.

decomposition of $X \sim I$ (reaction B) and that X and I are essential for electron transport to occur (see, for example, Chance, 1959).

Since it is assumed that dinitrophenol leads to the breakdown of $X \sim I$ (reaction B), it is apparent that at low concentrations of the phenol the rate at which electron transport occurs will be proportional to the rate at which X and I are provided. Under these conditions the succinate dehydrogenase has an obvious advantage in competing for cytochrome *b*.

Application of reaction kinetics to a scheme of the type

leads to the relationship

$$v = \frac{V_{\text{max}}[\text{dinitrophenol}]}{K_{\text{dinitrophenol}} + [\text{dinitrophenol}]}$$

The values of $K_{\text{dinitrophenol}}$ for dinitrophenol-stimulated adenosine triphosphatase, for dinitrophenol-stimulated malate and succinate oxidation, with both rat-liver and pigeon-heart mitochondria, were in the range 50–60 μM at pH 7.2 and 25° (obtained from Lineweaver-Burk plots) (see Chappell, 1963). These results indicate that there is only one site at which dinitrophenol acts and which is rate-limiting. Further, at low dinitrophenol concentrations it would be expected from the above equation that the rate of increase of the rate of respiration with dinitrophenol concentration would be directly proportional to its concentration, and that for various processes, e.g. NADH_2 and succinate oxidation, would be proportional to the maximum velocity of oxidation, V_{max} . Thus the ratio for the maximum rates of succinate and malate oxidation (Table 1) is of the order of 1.5, which is the observed ratio of the slopes of the rate of increase of the rate of respiration with dinitrophenol concentration for the two substrates. Dr R. W. Estabrook (personal communication) has obtained similar results by comparing the rates of succinate and β -hydroxybutyrate oxidation.

Another contributory feature may be the high level of the process of reversed electron transport from cytochrome *b* to NAD (see Chance & Holmberg, 1960). The steady-state level of oxidized nicotinamide nucleotide coenzyme may be expected to control the extent to which malate could be oxidized. In Fig. 10 an experiment is shown in which the rate of ADP-stimulated respiration, the

P:O ratio and the extent of oxidation of nicotinamide nucleotide coenzyme on addition of ADP were determined in a suspension of liver mitochondria in the presence of succinate, glutamate and β -chlorovinylarsenious oxide. The arsenical compound was added to prevent the oxidation of glutamate and the consequent reduction of nicotinamide nucleotide. The effect of malonate on these particles was determined. Two features should be noted. First, as the malonate concentration was increased the extent of oxidation of nicotinamide nucleotide in the presence of ADP, that is during the active phase of respiration, was increased. Secondly, the P:O ratio also increased with malonate concentration, indicating that malate oxidation was contributing to the overall change. This is also indicated by the Amytal sensitivity of ADP-stimulated succinate oxidation in the presence of malonate. A separate experiment, under otherwise identical conditions, revealed that, after the consumption of 0.48 $\mu\text{g. atom}$

Fig. 10. Effect of variation of malonate concentration on the rate of oxidation of succinate (○) by rat-liver mitochondria. The medium contained 10 mM-succinate, 10 mM-glutamate, 5 mM- P_i and $\mu\text{g.}$ of β -chlorovinylarsenious oxide/2 ml. The ADP-stimulated rates of succinate oxidation in the presence of 1.8 mM-Amytal (●) are also shown. P:O ratios (no Amytal present) (△) were determined by the method of Chance & Williams (1955). Simultaneously, the redox state of the bound nicotinamide nucleotide in state 3 (▲) was obtained from the steady-state readings in the various metabolic states, thus: oxidation (%)

$$= \frac{\text{State 4} - \text{state 3}}{\text{State 4} - \text{state 2}} \quad (\text{see Chance \& Williams 1955}).$$

of oxygen/ml. of suspension, 0.42 μ mole of malate had accumulated in the absence of malonate and only 0.15 μ mole in the presence of 0.5 mM-malonnate. However, there was only a 15 % increase in the oxidation of nicotinamide nucleotide at 0.25 mM-malonnate, a concentration of the inhibitor that produced a very marked increase in the Amytal sensitivity of succinate oxidation (about 45 % inhibition), which makes it likely that competition for cytochrome *b*, rather than the steady-state level of oxidized nicotinamide nucleotide, was the major cause of the inhibition of malate oxidation.

Greengard, Minnaert, Slater & Betel (1959) have shown that when rat-liver mitochondria oxidized succinate at concentrations less than 30 mM, in the presence of hexokinase and glucose, significant oxidation beyond the malate stage occurred. At higher succinate concentrations, or with rat-heart mitochondria, oxidation of malate derived from succinate did not occur to any extent. These findings are the opposite of those reported in this paper, if it is assumed that mitochondria derived from pigeon and rat hearts behave in the same way. However, the findings of these workers may be explained readily in terms of the above hypothesis. Over the relatively long periods of observation (20–35 min.) used by Greengard *et al.* (1959) substantial amounts of malate and fumarate would accumulate. These compounds would be expected to act as malonate did in the experiment shown in Fig. 10, that is to reduce the activity of the succinate dehydrogenase by competitive inhibition, thus permitting malate oxidation to occur. Since no transaminating agent was added in their experiments it would be expected that low rates of oxidation, probably due to accumulation of oxaloacetate, would result. This appears to be the case since the value given by them at a concentration of 15 mM-succinate for rat-liver mitochondria at 25° was 0.05 μ g. atom/mg. of protein/min.; this value is to be compared with the rates of ADP-stimulated succinate oxidation obtained with the oxygen electrode of 0.16–0.18. Only at 60 mM-succinate did Greengard *et al.* (1959) obtain rates of respiration approaching the latter value. That malate oxidation and the consequent production of oxaloacetate reduced the rate of succinate oxidation with their heart mitochondria is supported by their finding that Amytal caused a 60–100 % increase in the rate of respiration, even at high succinate concentrations.

The results described in this paper are consistent with the hypothesis that the rate-limiting step in some mitochondrial oxidations is the rate at which ATP can be synthesized. In other cases, dehydrogenase activity is limiting. With glutamate or α -oxoglutarate as substrates the conversion of the

latter compound into succinate appears to be the slowest reaction and with D- β -hydroxybutyrate oxidation the dehydrogenase is limiting, but apparently only at temperatures below 38° (Table 2).

SUMMARY

1. With rat-liver mitochondria glutamate is oxidized mainly by the transaminase pathway of Krebs & Bellamy (1960). However, in the presence of malonate or 3-fluoromalate, glutamate is oxidized through the dehydrogenase pathway. In the presence of 3-fluoromalate the rate is 50 % of that of the normal observed rate of adenosine diphosphate-stimulated glutamate oxidation. The rate-limiting reaction in the oxidation of glutamate by rat-liver and pigeon-heart mitochondria is possibly the rate at which α -oxoglutarate is converted into succinate, when oxidation occurs through the transaminase pathway, and the glutamate dehydrogenase when oxidation occurs through the dehydrogenase pathway.

2. The rate of oxidation of succinate or of malate in the presence of glutamate appears to be limited by the rate at which adenosine triphosphate can be synthesized. 2,4-Dinitrophenol caused higher rates of oxygen uptake than adenosine diphosphate and phosphate, with these substrates.

3. The extent to which the malate derived from succinate oxidation is further metabolized with both liver and pigeon-heart mitochondria depends on both the activity of the succinate dehydrogenase and the rate of flux of electrons through the respiratory chain. At low dinitrophenol concentrations, or with liver mitochondria with adenosine diphosphate, the malate was not oxidized to any significant extent. At higher dinitrophenol concentrations, and with pigeon-heart mitochondria with adenosine diphosphate, the malate derived from succinate oxidation was oxidized. In the absence of suitable systems for removal of oxaloacetate this resulted in an inhibition of succinate oxidation.

I thank Dr Britton Chance for the facilities he has provided for a part of this investigation.

REFERENCES

- Azzone, G. F. & Ernster, L. (1960). *Nature, Lond.*, **187**, 65.
Bonner, W. D. (1954). *Biochem. J.* **58**, 274.
Borst, P. & Slater, E. C. (1960). *Biochim. biophys. Acta*, **41**, 170.
Borst, P. & Slater, E. C. (1961). *Biochim. biophys. Acta*, **48**, 362.
Brierley, G. P., Bachmann, E. & Green, D. E. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1928.
Chance, B. (1959). In *Ciba Found. Symp.: Regulation of Cell Metabolism*, p. 91. Ed. by Wolstenholme, G. E. W. & O'Connor, G. M. London: J. and A. Churchill Ltd.

- Chance, B. & Conrad, H. (1959). *J. biol. Chem.* **234**, 1568.
- Chance, B. & Hagihara, B. (1961). *Proc. 5th int. Congr. Biochem., Moscow*, Symp. no. 5.
- Chance, B. & Hollunger, G. (1960). *Nature, Lond.*, **185**, 666.
- Chance, B. & Williams, G. R. (1955). *Advanc. Enzymol.* **17**, 65.
- Chappell, J. B. (1961a). In *Biological Structure and Function*, vol. 2, p. 71. Ed. by Goodwin, T. W. & Lindberg, O. London: Academic Press (Inc.) Ltd.
- Chappell, J. B. (1961b). *Fed. Proc.* **20**, 50.
- Chappell, J. B. (1962). *Biochem. J.* **84**, 62r.
- Chappell, J. B. (1963). *J. biol. Chem.* **238**, 410.
- Chappell, J. B. (1964). *Biochem. J.* **90**, 225.
- Chappell, J. B., Cohn, M. & Greville, G. D. (1963). In *Energy-Linked Functions of Mitochondria*, p. 219. Ed. by Chance, B. New York: Academic Press Inc.
- Chappell, J. B. & Greville, G. D. (1961). *Nature, Lond.*, **190**, 502.
- Chappell, J. B. & Greville, G. D. (1963a). *Symp. Biochem. Soc.* **23**, 39.
- Chappell, J. B. & Greville, G. D. (1963b). *Fed. Proc.* **22**, 526.
- Gal, E. M. (1960). *Arch. Biochem. Biophys.* **90**, 278.
- Greengard, P., Minnaert, K., Slater, E. C. & Betel, I. (1959). *Biochem. J.* **73**, 637.
- Krebs, H. A. & Bellamy, D. (1960). *Biochem. J.* **75**, 523.
- Lardy, H. A., Johnson, D. & McMurray, W. C. (1958). *Arch. Biochem. Biophys.* **78**, 587.
- Lardy, H. A. & Wellman, H. (1952). *J. biol. Chem.* **195**, 215.
- Lehninger, A. L. & Greville, G. D. (1953). *Biochim. biophys. Acta*, **12**, 188.
- Lehninger, A. L., Rossi, C. S. & Greenawalt, J. W. (1963a). *Biochem. biophys. Res. Commun.* **6**, 444.
- Lehninger, A. L., Rossi, C. S. & Greenawalt, J. W. (1963b). *Fed. Proc.* **22**, 526.
- Pardee, A. B. & Potter, V. R. (1948). *J. biol. Chem.* **176**, 1085.
- Singer, T. P. & Kearney, E. B. (1956). *Arch. Biochem. Biophys.* **61**, 397.
- Slater, E. C. & Hülsmann, W. C. (1959). In *Ciba Found. Symp.: Regulation of Cell Metabolism*, p. 58. Ed. by Wolstenholme, G. E. W. & O'Connor, G. M. London: J. and A. Churchill Ltd.

Biochem. J. (1964) **90**, 248

Preliminary Studies on the Isolation and Metabolism of an Intermediate in Aromatic Biosynthesis: Chorismic Acid

By MARGARET I. GIBSON AND F. GIBSON

Bacteriology School, University of Melbourne, Parkville, N. 2, Victoria, Australia

(Received 11 April 1963)

It has been suggested (Gibson, Gibson, Doy & Morgan, 1962) that the pathway of biosynthesis of the aromatic amino acids, tryptophan, phenylalanine and tyrosine, and the aromatic growth factors, 4-aminobenzoic acid and 4-hydroxybenzoic acid, may be as outlined in Scheme 1.

This scheme was based particularly on the observations that 3-enolpyruvylshikimic acid 5-phosphate is a likely precursor for the three aromatic amino acids (Levin & Sprinson, 1960; Gibson *et al.* 1962; Rivera & Srinivasan, 1962) and that mutants have been described (Davis & Mingioli 1953) which accumulate 3-enolpyruvylshikimic acid and require for growth phenylalanine, tyrosine, tryptophan, 4-aminobenzoic acid and 4-hydroxybenzoic acid. From the latter observation it may be deduced that there is at least one further common intermediate beyond 3-enolpyruvylshikimic acid 5-phosphate. This is the 'compound X' of Scheme 1. If such a compound exists, a mutation affecting an enzyme concerned in converting compound X into prephenic acid should result in an auxotroph requiring phenylalanine and tyrosine. Such double requirements have been reported

(Davis & Mingioli, 1953; Schwinck & Adams, 1959), but examination of the auxotrophs has shown that their requirements are due either to partial blocks further back than compound X or to two separate mutations affecting the conversion of prephenic acid into phenylpyruvic acid on the one hand and into 4-hydroxyphenylpyruvic acid on the other.

The present paper concerns the search for the hypothetical compound X. Study of the reactions in the region of compound X is complicated by the fact that blocking one reaction results in shunting of the intermediates along alternative pathways (Morgan, Gibson & Gibson, 1962). By imposing subsequent mutations affecting each pathway in turn it was hoped to obtain cell extracts that would accumulate compound X itself. To this end a strain of *Aerobacter aerogenes* (T17) was used, cell-free extracts of which had previously been studied for their ability to carry out the conversion of shikimic acid into anthranilic acid or the phenylpyruvic acids (Morgan *et al.* 1962; Morgan, Gibson & Gibson, 1963). In the present work this strain was irradiated and further mutants were isolated with additional metabolic lesions affecting the phenyl-