- Potter, V. R. & Recknagel, R. O. (1951). In *Phosphorus Metabolism*, vol. 1, p. 377. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
- Potter, V. R., Siekevitz, P. & Simonson, H. C. (1953). *J. biol. Chem.* **205**, 893.
- Robertson, H. E. & Boyer, P. D. (1955). *J. biol. Chem.* **214**, 295.
- Sacktor, B. (1953). *J. gen. Physiol.* **36**, 371.
- Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
- Siekevitz, P. & Potter, V. R. (1955). *J. biol. Chem.* **215**, 221.
- Slater, E. C. (1949). *Biochem. J.* **45**, 1.
- Slater, E. C. (1953). *Biochem. J.* **53**, 157.
- Slater, E. C. (1956). *Proc. 3rd Int. Congr. Biochem., Brussels*, 1955, p. 264.
- Slater, E. C. & Cleland, K. W. (1953). *Biochem. J.* **55**, 566.
- Sumner, J. B. (1944). *Science*, **100**, 413.
- Swanson, M. A. (1956). *Biochim. biophys. Acta*, **20**, 85.
- Witter, R. F., Watson, M. L. & Cottone, M. A. (1955). *J. biophys. biochem. Cytol.* **1**, 127.

The Enzymic Hydrolysis of Adenosine Triphosphate by Liver Mitochondria

2. EFFECT OF INHIBITORS AND ADDED COFACTORS

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In the previous paper (Myers & Slater, 1957*a*), experiments were reported which were interpreted on the basis that four different enzymes or enzyme systems were responsible for the hydrolysis of adenosine triphosphate (ATP) by liver mitochondria, under various conditions. Each of these systems appeared to be relatively specific for ATP. Three of the four enzyme systems were activated by dinitrophenol (DNP), which suggested that they contained components which might be involved in oxidative phosphorylation.

In the present paper, the effect of various inhibitors of the respiratory chain on the hydrolysis of ATP is examined. This investigation was undertaken in order to determine whether any of the members of the respiratory chain are directly involved in the enzymic hydrolysis of ATP by mitochondrial preparations, as has been suggested in some formulations of oxidative phosphorylation (e.g. Slater, 1953*a*).

A preliminary report of some of these results has appeared (Myers & Slater, 1957*b*).

METHODS

All the methods have been described in the previous paper (Myers & Slater, 1957*a*).

The diphosphopyridine nucleotide (DPN) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and the reduced diphosphopyridine nucleotide (DPNH) was prepared by reduction with alcohol dehydrogenase, as described by Slater (1953*b*).

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RESULTS

Cyanide. Chance & Williams (1956) have shown that freshly prepared liver mitochondria contain endogenous substrate, which is rapidly exhausted by the addition of phosphate acceptor or an uncoupling agent such as DNP in the presence of oxygen. One would expect that under the conditions of the measurement of the ATPase activity in this paper, the endogenous substrate would be rapidly exhausted and that all the components of the respiratory chain would be completely oxidized for the greater part of the assay. So far as the cytochrome system was concerned, this was confirmed by direct spectroscopic observation. The addition of 10^{-3} M-KCN prevented the oxidation of endogenous substrate, and maintained the cytochromes (and presumably also the pyridine nucleotides and flavoproteins) in the reduced state during the whole course of the assay. However, Fig. 1*A* shows that this addition had no effect on the ATPase activity throughout the range of pH values studied.

Similarly, the addition to the Keilin & Hartree (1947) heart-muscle preparation of 10^{-3} M-KCN, either alone or in the presence of 0.01 M-succinate (added to reduce the respiratory chain, since this preparation is free from endogenous substrate), had no effect on the ATPase activity. It must be concluded therefore that neither cytochrome a_3 , which combines with cyanide, nor the other members of the respiratory chain which undergo oxidation and reduction under these conditions are likely to be involved in the hydrolysis of ATP by mitochondrial preparations.

Inhibitors of succinic dehydrogenase. The participation of succinic dehydrogenase in the hydrolysis of ATP by the Keilin & Hartree heart-muscle preparation was excluded by incubation with 0.02M-KCN for 3 hr. at 38° and pH 7.4. Under these conditions, succinic dehydrogenase is completely inactivated (Tsou, 1951). Nevertheless, there was no effect on the rate of hydrolysis of ATP at any pH value between 5 and 10. Further evidence that the succinic dehydrogenase is not involved in this reaction was afforded by lack of effect of 0.01M-malonnate on the ATPase activities of preparations of liver mitochondria.

Fig. 1. Effect of various inhibitors on the hydrolysis of ATP by liver mitochondria. Data are presented for the ATPase activities of normal mitochondria in the presence of 10^{-4}M-DNP (○) and of aged and frozen mitochondria (Myers & Slater, 1957a) (●); a significant stimulation of the ATP hydrolysis by normal mitochondria in the presence of 10^{-4}M-DNP is produced only between pH 6 and 8.5 (see Myers & Slater, 1957a). The following inhibitors were used: (A) 10^{-3}M-KCN ; (B) $10^{-3}\text{M-Na}_2\text{S}_2\text{O}_8$; (C) $0.01\text{M-guanidine hydrochloride}$; (D) $10^{-4}\text{M-p-chloromercuribenzoate}$; (E) 0.06M-KF ; (F) bovine plasma albumin in concentrations of 5, 3.3 and 10 mg./ml. from top to bottom. The reaction mixture with the normal mitochondria was: KCl (0.075M); sucrose (0.05M); tris buffer (0.05M); MgCl_2 (0.001M); ethylenediaminetetraacetic acid ($6 \times 10^{-4}\text{M}$); ATP (0.002M). With aged mitochondria the ethylenediaminetetraacetic acid was omitted.

Antimycin and 2:3-dimercaptopropanol. Both of these compounds can interrupt the respiratory chain at some point between cytochrome *b* and cytochrome *c* (Slater, 1949; Potter & Reif, 1952). A concentration of antimycin sufficient to stop respiration (10^{-6}M) had no significant effect on the hydrolysis of ATP by normal mitochondria in the presence of DNP or by aged and frozen mitochondria between pH 5 and 10. Higher concentrations of antimycin stimulated the hydrolysis of ATP by normal mitochondria in the absence of DNP. Incubation of the Keilin & Hartree heart-muscle preparation with $0.01\text{M-2:3-dimercaptopropanol}$ for 30 min. at 37° , a procedure sufficient to inactivate the respiratory chain completely (Slater, 1949), caused only a slight inactivation of the ATPase at all pH values.

Fig. 2. Hydrolysis of ATP by liver mitochondria after extraction of the cytochrome *c*, as described by Schneider *et al.* (1948). Data are given for the extracted mitochondria in the absence of DNP (+), in the presence of 10^{-4}M-DNP (○) and in the presence of $10^{-4}\text{M-DNP} + 10^{-4}\text{M-cytochrome c}$ (●). The same mitochondria were also assayed in the absence (Δ) and the presence (▲) of $10^{-4}\text{M-cytochrome c}$ after the mitochondrial preparation had been aged and frozen. The reaction mixtures were the same as in Fig. 1.

Addition and removal of cytochrome *c*. The hydrolysis of ATP by mitochondria in the presence or absence of DNP was scarcely affected by the addition of 7×10^{-5} M-cytochrome *c*, even after the mitochondria were washed twice with water at 0° and subsequently twice with 0.15 M-NaCl at 0° in order to extract the cytochrome *c* (Schneider, Claude & Hogeboom, 1948). Although the exposure to hypotonic conditions activated the latent ATPase activity to a considerable extent, the addition of DNP still stimulated the ATPase activity (Fig. 2). After this mitochondrial preparation was aged and frozen (Myers & Slater, 1957*a*), the pH-activity curve of the fully activated ATPases appeared to be the same as obtained with a similar preparation from normal mitochondria [cf. Fig. 2 with Fig. 8 of the previous paper (Myers & Slater, 1957*a*)]. The curve was not affected by the addition of 10^{-4} M-cytochrome *c*, either alone or in the presence of dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) in order to reduce the cytochrome *c*. The effect of dithionite itself on the ATPase activity will be described below.

Removal and addition of diphosphopyridine nucleotide and reduced diphosphopyridine nucleotide. Both aged liver mitochondria and the Keilin & Hartree heart-muscle preparation, which contain little DPN, exhibit a high ATPase activity, which was not affected by the addition of 10^{-4} M-DPN or DPNH. The same concentration of DPNH also had no effect on the ATPase activity of fresh liver mitochondria in the presence of DNP, between pH 5.5 and 8.5.

α -Tocopherol and vitamin K_1 . There are some indications that these two vitamins may also be involved in oxidative phosphorylation, either as members of the respiratory chain or as essential cofactors (Martius, 1956; Nason & Lehmann, 1956; Bouman & Slater, 1956). The Keilin & Hartree heart-muscle preparation contains an appreciable amount of tocopherol, but no vitamin K (Bouman & Slater, 1956). The ATPase activities of the Keilin & Hartree heart-muscle preparation were not affected by the addition of 0.07 mg. of DL- α -tocopherol or 0.02 mg. of vitamin K_1 /ml. of reaction mixture; the stable stock suspensions used in these experiments were prepared in sucrose solutions containing 10 % of ethanol and 0.5 % of plasma albumin (cf. Nason & Lehmann, 1956). The same amount of tocopherol also had no effect on the ATPase activities of normal liver mitochondria in the presence of DNP.

Azide. One of the most potent inhibitors of the ATPase activities of mitochondrial preparations is sodium azide (Meyerhof & Ohlmeyer, 1952; Novikoff, Hecht, Podber & Ryan, 1952; Sacktor, 1953; Robertson & Boyer, 1955; Maruyama, 1954, 1956). However, its action is rather complicated since it also uncouples oxidative phosphorylation (Loomis & Lipmann, 1949) and can stimulate as well as

inhibit the ATPase activity of normal liver mitochondria (Robertson & Boyer, 1955; Swanson, 1956). Our experiments showed that the stimulation of the ATPase activity by azide in the absence of DNP precedes the slower inhibitory reaction; the major portion of the ATP hydrolysis occurred within the first 5 min. after adding the normal liver mitochondria to a reaction mixture containing 10^{-3} M- NaN_3 . The slow rate of the inhibitory reaction could also be demonstrated by experiments in which the mitochondria were incubated with azide before the ATP was added (Fig. 3); the stimulation by the azide became much less after pre-incubation for 10 min. and disappeared completely when the mitochondria were pre-incubated for 20 min. The stimulation of the latent ATPase activity of normal mitochondria by 10^{-3} M- NaN_3 was maximal at about pH 6 (Fig. 4); this result is similar to that observed with low concentrations of DNP (Myers & Slater, 1957*a*).

The same concentration of azide produced a strong inhibition of the ATPase, activated by the addition of DNP to normal mitochondria or by ageing and freezing the mitochondrial suspensions (Fig. 1*B*). A similar inhibition was observed with heart sarcosomes and with the Keilin & Hartree heart-muscle preparation.

Calcium. Low concentrations of Ca^{2+} ions cause a reversible uncoupling of oxidative phosphorylation, and higher concentrations result in a number of irreversible effects (Potter, Siekevitz & Simonson, 1953; Chance & Williams, 1956). As would be

Fig. 3. Effect of sodium azide on the hydrolysis of ATP by normal liver mitochondria. The initial reaction mixture contained 0.075 M-KCl, 0.108 M-sucrose, 0.001 M-ethylenediaminetetra-acetic acid, 0.003 M- MgCl_2 , pH 7.4. p (NaN_3) indicates the negative logarithm of the molar concentration of NaN_3 added. The mitochondria were pre-incubated in these reaction mixtures with or without azide for (A) 0, (B) 10 and (C) 20 min. before adding a small volume of ATP solution (final concn.: 0.002 M-ATP). The control activities in the absence of azide are indicated by the horizontal broken lines.

expected, low concentrations of Ca^{2+} ions (10^{-3}M) also stimulated the latent ATPase activity of normal mitochondria (cf. Lardy & Wellman, 1953; Potter *et al.* 1953), which was maximal at about pH 6.5 (Fig. 4). The same concentration inhibited the ATPase activity of normal mitochondria at high pH values, and a similar inhibition above pH 9 was also observed with mitochondria, fully activated by ageing followed by freezing and thawing. This inhibition at high pH, which was also obtained with Mg^{2+} ions (Myers & Slater, 1957*a*), is probably due to combination with the ATP (Martell & Schwarzenbach, 1956).

p-Chloromercuribenzoate. This compound is a potent inhibitor of the mitochondrial ATPase at

Fig. 4. Stimulation of the ATPase activities of normal liver mitochondria by various compounds. The reaction mixture contained 0.075M-KCl, 0.05M-sucrose, 0.05M-tris buffer, 0.002M-ATP, 0.001M- MgCl_2 and 0.0006M-ethylenediaminetetra-acetic acid. The ATPase activities were measured in the presence of 10^{-3}M-CaCl_2 (Δ), 10^{-3}M-NaN_3 (●) and $10^{-4}\text{M-p-chloromercuribenzoate}$ (+), the enzymic reaction being allowed to proceed for 15 min. in the first case and for 5 min. in the second and third cases. The control activities in the absence of these compounds were similar in these experiments and the average values obtained are shown above (○).

pH 7.4 (Novikoff *et al.* 1952; Sacktor, 1953; Lardy & Wellman, 1953; Hunter, 1956) but, like azide, can also activate the latent ATPase activity of normal mitochondria (Hunter, 1956). However, the activation occurred at all pH values from 5.5 to 9 (Fig. 4) and was not restricted to the lower pH values as was found with low concentrations of DNP, azide and Ca^{2+} ions. Alterations in the translucency of the normal mitochondrial suspensions were also observed (cf. Hunter, Davis & Corlat, 1956). The stimulation of the latent ATPase activity by *p*-chloromercuribenzoate may therefore be due to a general damage of the mitochondrial structure rather than to a specific uncoupling action.

The degree of inhibition of the activated ATPase by *p*-chloromercuribenzoate was highly dependent on the Mg^{2+} ions and salt concentrations in the reaction medium and on the state of the mitochondria (Lardy & Wellman, 1953). The ATPase of normal mitochondria in the presence of DNP was strongly inhibited by $10^{-4}\text{M-p-chloromercuribenzoate}$ between pH 6 and 8, but a stimulation was observed in the region of pH 9 (Fig. 1*D*), where DNP has little effect on the latent ATPase activity but where the *p*-chloromercuribenzoate itself also stimulated in the absence of DNP (cf. Fig. 4). The same concentration of *p*-chloromercuribenzoate had little effect on the hydrolysis of ATP by mitochondria activated by ageing followed by freezing and thawing (Fig. 1*D*). A higher concentration (10^{-3}M) also had little effect. This might suggest that the hydrolysis of ATP by normal mitochondria in the presence of DNP follows a different pathway from the hydrolysis by aged and frozen mitochondria; however, the lack of inhibition by *p*-chloromercuribenzoate in the latter case could be due to the liberation of sulphhydryl groups during the process of ageing, and a consequent protection of the ATPases against the inhibitory action. The ATPase activities of the Keilin & Hartree heart-muscle preparation were inhibited 40–60% by $10^{-3}\text{M-p-chloromercuribenzoate}$.

Fluoride. This compound is a strong inhibitor of the pyrophosphatase and adenylkinase (Barkulis & Lehninger, 1951; Swanson, 1952; Sacktor, 1953; Siekevitz & Potter, 1953), but has less effect on the ATPase activity of liver mitochondria at pH 7.4 (Kielley & Kielley, 1951; Novikoff *et al.* 1952; Sacktor, 1953; Lardy & Wellman, 1953; Potter *et al.* 1953). However, the hydrolysis of ATP by normal mitochondria in the presence of DNP could be inhibited by high concentrations of KF (0.06M) between pH 6 and 8; as with *p*-chloromercuribenzoate, an activation was observed at higher pH values (Fig. 1*E*). On the other hand, 0.06M-KF had little effect on the hydrolysis of ATP by aged and frozen mitochondria (Fig. 1*E*), aged mitochondria or the Keilin & Hartree heart-muscle preparation.

We did not observe either an uncoupling of oxidative phosphorylation (cf. Middlebrook & Szent-Györgyi, 1955) or a stimulation of the latent ATPase activity of normal mitochondria by high concentrations of KBr and KI. On the other hand, high concentrations of KF did exert some uncoupling action (cf. Kielley & Kielley, 1951) and also stimulated the latent ATPase activity of normal mitochondria in the absence of DNP. However, an appreciable stimulation was observed only in the absence of Mg^{2+} ions, possibly because the mitochondria are then more fragile. The concentration of fluoride (about 0.01 M) used by Copenhaver & Lardy (1952) and most other investigators in experiments on oxidative phosphorylation, had no significant effect on the latent ATPase activity of normal mitochondria in the presence of 0.001 M- $MgCl_2$ and 0.0006 M-ethylenediaminetetra-acetic acid.

Guanidine. Hollünger (1955) has shown that guanidine inhibits the hydrolysis of ATP by aged mitochondria at pH 7.4 but has little effect on the hydrolysis by normal mitochondria in the presence of DNP. These observations were confirmed over the whole pH range in the present investigation, with 0.01 M-guanidine (Fig. 1C). These results might also indicate different pathways for the ATPase activities in normal and aged preparations. However, the ATPase activity of normal mitochondria in the presence of DNP can be inhibited by higher concentrations of guanidine.

Albumin. The effect of plasma albumin was of particular interest because it is reported to combine with mitochrome, a haemoprotein which is liberated from aged mitochondria, and it uncouples oxidative phosphorylation and stimulates the latent ATPase activities of normal mitochondria in the same way as DNP; both effects are prevented by the addition of serum albumin (Shmukler & Polis, 1953; Polis & Shmukler, 1954).

No evidence could be obtained to suggest that mitochrome played a role in the activity of the ATPase of our aged and frozen suspensions of liver mitochondria. Bovine plasma albumin (fraction V from Armour Laboratories) in concentrations up to 10 mg./ml. (see Fig. 1) and ovalbumin (up to 50 mg./ml.) had no effect. Mitochondrial fragments obtained in such a way as to extract soluble proteins, by the procedure of Shmukler & Polis (1953), still possessed a high ATPase activity, which was not increased by adding the 'soluble' fraction (see Table 1). The high activity of the Keilin & Hartree preparation and of the mitochondrial fragments of Kielley & Kielley (1953) also speak against an easily soluble enzyme being concerned in these activities, since soluble enzymes are removed from these preparations.

Albumin had no effect on the stimulation of the ATPase by azide, but markedly decreased the

activity in the presence of DNP (Fig. 1F). However, the significance of this apparent inhibition is uncertain since the inhibition was maximum at pH 9.4, where DNP no longer stimulates. Albumin does not prevent the uncoupling action of DNP (Slater & Lewis, 1954).

Dithionite. Dithionite caused a marked activation of the ATPase activity of aged and frozen mitochondria between pH 6 and 9 (Fig. 5). The degree of stimulation by 10^{-3} M- $Na_2S_2O_4$ was maximum at pH 7.5, and negligible above 9; it was not increased when the concentration of dithionite was increased to 10^{-2} M. The activation by 10^{-3} M- $Na_2S_2O_4$ was not affected by the addition of catalase, indicating that it was not caused by H_2O_2 produced by the auto-oxidation of $Na_2S_2O_4$. There was no stimulation of the ATPase activity of normal mitochondria in the presence of 10^{-4} M-DNP; however, this experiment is not conclusive since the $Na_2S_2O_4$ altered the colour of the DNP from yellow to orange.

Other reducing agents did not give the same effect as $Na_2S_2O_4$. Thus 10^{-3} M- $K_4Fe(CN)_6$ brought about only a slight activation, and 10^{-3} M-glutathione (cf. Novikoff *et al.* 1952) and 10^{-3} M-ascorbic acid were slightly inhibitory (Fig. 5). An oxidizing agent, 10^{-3} M- $K_3Fe(CN)_6$, also had no effect on the ATPase activities of aged and frozen mitochondria.

Table 1. *Enzymic hydrolysis of adenosine triphosphate by the soluble and insoluble fractions of aged liver mitochondria*

Normal liver mitochondria were suspended in 0.15 M-KCl + 0.02 M- $NaHCO_3$ and aged by incubation for 80 min. at 30°. The 'soluble' fraction was recovered by centrifuging off the mitochondria at 27 000 g. The insoluble mitochondrial material was washed with the salt solution, resuspended and incubated for a further 80 min. at 30° to complete the extraction of soluble proteins. The mixture was again centrifuged at 27 000 g, and the sediment washed and resuspended. This constituted the insoluble fraction. The reaction mixture for the ATPase assay contained 0.075 M-KCl, 0.05 M-sucrose, 0.05 M-tris buffer, 0.002 M-ATP and 0.001 M- $MgCl_2$.

pH	ATPase activity (μmoles of P/mg. of protein/hr.)		ATPase activity of the soluble and insoluble fractions combined (calc. as % of the sum of their separate activities)
	Insoluble fraction	'Soluble' fraction	
5.5	10.7	2.9	94
6.0	15.9	2.6	93
6.5	18.7	2.6	95
7.0	22.3	2.8	92
7.5	25.2	3.0	90
8.0	30.1	3.7	86
8.5	34.0	4.1	81
9.0	32.1	3.7	83
9.5	27.8	2.7	95
10.0	22.0	1.7	96

Fig. 5. Effect of reducing agents on the ATPase activities of aged and frozen liver mitochondria. The reaction mixture is the same as that given in Table 1. ATPase activities were measured in the absence of further addition (○) and in the presence of 10^{-3} M-sodium dithionite (●), 10^{-3} M-glutathione (Δ) and 10^{-3} M-ascorbic acid (+).

DISCUSSION

The coupling of phosphorylation with the transfer of hydrogen (or electrons) through the respiratory chain has been formulated in its simplest terms by the reactions (1) and (2):

where AH_2 and B are adjacent members of the respiratory chain, whose interaction is coupled with phosphorylation, and C is an intermediate linking the oxidation-reduction with the phosphorylation reaction (Slater, 1953a). It was postulated that

DNP was in some way able to bring about the hydrolysis of $\text{A} \sim \text{C}$ [reaction (3)]:

The hydrolysis of ATP stimulated by DNP is brought about by the reverse of reaction (2), followed by reaction (3).

According to this formulation, a member of the respiratory chain (A), as well as a component (C), which does not undergo oxidation-reduction, is directly involved in the hydrolysis of ATP. Therefore combination of A with an inhibitor or its reduction to AH_2 would be expected to inhibit the ATPase activity. In this paper it has been shown that the addition of inhibitors of the respiratory chain (cyanide, antimycin, malonate), or the removal or inactivation of certain components (DPN, cytochrome c, the 2:3-dimercaptopropanol-labile factor), or the reduction of all components of the respiratory chain by the addition of cyanide and substrate, did not cause any inhibition of the ATPase activity. Thus the minimum hypothesis represented by equations (1), (2) and (3) is no longer adequate, and must be modified to take A out of the 'ATPase' reaction. The simplest extension of the theory is to introduce an additional step, with an additional component, between reactions (1) and (2). Chance & Williams (1956) have also given kinetic reasons for introducing an additional component (X) between reactions (1) and (2). They have also pointed out that C in the above scheme has the properties of an inhibitor, since $\text{A} \sim \text{C}$ is an inhibited form of A which cannot be reduced in the respiratory chain to AH_2 . For this reason, Chance & Williams have replaced C by I (inhibitor). Since it is clearly desirable to avoid a multiplicity of symbols, we shall adopt the symbols X and I of Chance & Williams. We shall not, however, replace AH_2 and B by specific members of the respiratory chain, because we do not think that these have been definitely identified. The modified hypothesis then becomes:

The hydrolysis of ATP is now explained by the reverse of reaction (3), followed by reaction (4). The latter reaction might involve DNP-I as an intermediate (Chance & Williams, 1956).

It is probable that there are three oxidation-reduction steps in the respiratory chain which are coupled with phosphorylation. It seems likely that each of the oxidation-reduction pairs (AH_2 and B)

involved would require a different mediator I. Thus there would be three different inhibitors which could bring about the hydrolysis of ATP in the presence of DNP by the reversal of reaction (3), followed by reaction (4). It is possible that the three different DNP-sensitive enzyme systems suggested in the previous paper (Myers & Slater, 1957*a*) are reactions involving the three different inhibitors. In the absence of evidence to the contrary, it is simplest to assume that X is the same in all cases and could, in fact, represent a general enzyme or coenzyme of respiratory-chain phosphorylation.

Assuming that the three peaks obtained under different conditions with DNP represent three different enzymes, all three are inhibited by azide, which suggests that they possess a common component which could be X. A sensitivity to azide naturally raises the possibility that a haematin compound is involved, although against this it should be noted that the inhibition was a slow reaction in contrast with the rapid reaction usually occurring between haematin compounds and azide. It is possible that X is mitochrome (Polis & Shmukler, 1954), but all attempts to demonstrate its action in our preparations failed. However, we must await further details of the preparation and properties of mitochrome before we can settle this point.

The ATPase activity at pH 9.4 is also inhibited by azide, although it is not stimulated by DNP. One possibility is that this enzyme involves X, but not I. It would be expected that reaction (3) of the new scheme would occur in two steps, e.g.:

Some reaction of this type appears to be necessary to explain the rapid exchange between the oxygen atoms of inorganic phosphate and water catalysed by liver mitochondria (Cohn, 1953; Cohn & Drysdale, 1955; Boyer, Falcone & Harrison, 1954; Robertson & Boyer, 1955; Luchsinger, Falcone & Reynard, 1955). The hydrolysis of ATP and the lack of any effect of DNP would be explained if, at pH 9.4, $X \sim P$ reacts with water much more rapidly than with any of the inhibitors.

The mechanism of the stimulation of the latent ATPase activity by ageing and freezing the mitochondria is not clear, but the pH-activity curves suggest that the same enzymes (and therefore the same inhibitors) are involved. It seems likely then that hydrolysis of $X \sim I$ also occurs with these preparations.

SUMMARY

1. The addition to liver mitochondria of inhibitors of the respiratory chain (cyanide, anti-

mycin, malonate), or the removal or inactivation of certain components (diphosphopyridine nucleotide, cytochrome *c*, the 2:3-dimercaptopropanol-labile factor), or the reduction of all components of the respiratory chain by the addition of cyanide and substrate, did not cause any inhibition of the adenosine triphosphatase activity. It appears very unlikely then that a member of the respiratory chain is directly involved in the hydrolysis of adenosine triphosphate (ATP).

2. When azide was added to liver mitochondria, the rate of hydrolysis of ATP was first stimulated (particularly at pH 6) and then inhibited. In the presence of dinitrophenol, the hydrolysis of ATP was inhibited over the whole pH range studied (5.5–10).

3. Calcium stimulated at acid pH values (maximum at pH 6.5) and inhibited at higher pH values.

4. *p*-Chloromercuribenzoate activated the ATPase at all pH values between 5.5 and 9, probably because of a general damage of the mitochondrial structure. In the presence of dinitrophenol the mercurial inhibited between pH 6 and 8 but stimulated at pH 9.

5. Fluoride has little effect on the ATPase activity of aged and frozen mitochondria, but in the presence of dinitrophenol inhibited the activity of normal mitochondria between pH 6 and 8, and stimulated at pH 9.

6. Guanidine, on the other hand, inhibited the systems in aged and frozen mitochondria more than those in normal mitochondria activated by dinitrophenol.

7. Albumin had no effect on the ATPase activity of aged and frozen mitochondria, or on mitochondria activated by azide, but markedly inhibited the activity in the presence of dinitrophenol, especially at alkaline pH values.

8. Dithionite strongly activated the ATPase of aged and frozen mitochondria, with a maximum at pH 7.5.

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REFERENCES

- Barkulis, S. S. & Lehninger, A. L. (1951). *J. biol. Chem.* **190**, 339.
- Bouman, J. & Slater, E. C. (1956). *Nature, Lond.*, **177**, 1181.
- Boyer, P. D., Falcone, A. B. & Harrison, W. H. (1954). *Nature, Lond.*, **174**, 401.
- Chance, B. & Williams, G. R. (1956). *Advanc. Enzymol.* **17**, 65.
- Cohn, M. (1953). *J. biol. Chem.* **201**, 735.
- Cohn, M. & Drysdale, G. R. (1955). *J. biol. Chem.* **216**, 831.
- Copenhaver, J. A. & Lardy, H. A. (1952). *J. biol. Chem.* **195**, 225.
- Höllinger, G. (1955). *Acta pharm. tox., Kbh.*, **11**, suppl. 1.

- Hunter, F. E. (1956). *Proc. 3rd Int. Congr. Biochem., Brussels*, 1955, p. 298.
- Hunter, F. E., Davis, J. & Corlat, L. (1956). *Biochim. biophys. Acta*, **20**, 237.
- Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 500.
- Kielley, W. W. & Kielley, R. K. (1951). *J. biol. Chem.* **191**, 485.
- Kielley, W. W. & Kielley, R. K. (1953). *J. biol. Chem.* **200**, 213.
- Lardy, H. A. & Wellman, H. (1953). *J. biol. Chem.* **201**, 357.
- Loomis, W. F. & Lipmann, F. (1949). *J. biol. Chem.* **179**, 503.
- Luchsinger, W. W., Falcone, A. B. & Reynard, A. M. (1955). *Fed. Proc.* **14**, 247.
- Martell, A. E. & Schwarzenbach, G. (1956). *Helv. chim. acta*, **39**, 653.
- Martius, C. (1956). *Proc. 3rd Int. Congr. Biochem., Brussels*, 1955, p. 1.
- Maruyama, K. (1954). *Arch. Biochem. Biophys.* **52**, 485.
- Maruyama, K. (1956). *Arch. Biochem. Biophys.* **60**, 74.
- Meyerhof, O. & Ohlmeyer, P. (1952). *J. biol. Chem.* **195**, 11.
- Middlebrook, M. & Szent-Györgyi, A. (1955). *Biochim. biophys. Acta*, **18**, 407.
- Myers, D. K. & Slater, E. C. (1957a). *Biochem. J.* **67**, 558.
- Myers, D. K. & Slater, E. C. (1957b). *Nature, Lond.*, **179**, 363.
- Nason, A. & Lehmann, I. R. (1956). *J. biol. Chem.* **222**, 511.
- Novikoff, A. B., Hecht, L., Podber, E. & Ryan, J. (1952). *J. biol. Chem.* **194**, 153.
- Polis, B. D. & Shmukler, H. W. (1954). *Abstr. 126th Meeting Amer. Chem. Soc.* p. 72c.
- Potter, V. R. & Reif, A. E. (1952). *J. biol. Chem.* **194**, 287.
- Potter, V. R., Siekevitz, P. & Simonson, H. C. (1953). *J. biol. Chem.* **205**, 893.
- Robertson, H. E. & Boyer, P. D. (1955). *J. biol. Chem.* **214**, 295.
- Sacktor, B. (1953). *J. gen. Physiol.* **36**, 371.
- Schneider, W. C., Claude, A. & Hogeboom, G. H. (1948). *J. biol. Chem.* **172**, 451.
- Shmukler, H. W. & Polis, B. D. (1953). *Abstr. 124th Meeting Amer. Chem. Soc.* p. 13c.
- Siekevitz, P. & Potter, V. R. (1953). *J. biol. Chem.* **200**, 187.
- Slater, E. C. (1949). *Biochem. J.* **45**, 14.
- Slater, E. C. (1953a). *Nature, Lond.*, **172**, 975.
- Slater, E. C. (1953b). *Biochem. J.* **53**, 157.
- Slater, E. C. & Lewis, S. E. (1954). *Biochem. J.* **58**, 337.
- Swanson, M. A. (1952). *J. biol. Chem.* **194**, 685.
- Swanson, M. A. (1956). *Fed. Proc.* **15**, 367.
- Tsou, C. L. (1951). *Biochem. J.* **49**, 512.

A Comparison of the Properties of Mitochondria Isolated from Liver and Heart

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It is now well established that in all animal cells which have been examined the mitochondria are the site of the main intracellular respiratory enzyme systems which are responsible for the synthesis of adenosine triphosphate (ATP). The uniformity of structure, composition and properties of mitochondria isolated from a wide variety of cells is most striking. Nevertheless, some differences might be expected between mitochondria of tissues which differ so much in form and function as the liver and heart muscle. A careful study and correlation of the differences between the mitochondria from the two tissues might possibly throw light on the biochemical mechanisms in the two granules.

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Chance (1956) has recently drawn attention to some differences between the properties of liver mitochondria, as isolated in his laboratory, and heart-muscle mitochondria (sarcosomes) isolated by ourselves. Chance does not consider the possibility that these differences reflect a true difference between the mitochondria in the two tissues. On the contrary, he concludes that the differences are due to the fact that the sarcosomes which we used have been damaged in the course of their preparation.

It is the purpose of the present paper to compare in some detail the properties of the mitochondria isolated from the two tissues. The heart sarcosomes are indeed qualitatively similar to the liver mitochondria, but quantitative differences were observed. An attempt has been made to determine which of these differences can be ascribed to damage