more toxic by intraperitoneal injection than any of the simple peroxides with which it was compared. Autoxidized methyl linoleate was less toxic than most of the simple peroxides.

5. The LD_{50} of autoxidized linoleic acid (0·26 μ-mole of peroxide/g.) was only slightly higher than the mean increase of peroxide previously found in mice after 950r. of X-rays (0·22 μmole/g.).

6. Injected peroxides kill more quickly than radiation, but this may be a question of distribution.

7. The evidence is consistent with the view that radiation toxicity is due to initiation of chain autoxidation of essential fatty acids producing lethal doses of peroxides in sites not reached by vitamin E.

We wish to thank Dr John H. Heller for highly purified squalene, Dr R. Laterjet for disuccinoyl peroxide, Mr M. J. Corp for X-radiation, Mr R. T. Beadle and Mr J. W. P. Phelpstead for efficient technical assistance, and the animal-house staff for care of the animals.

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The Enzymic Hydrolysis of Adenosine Triphosphate by Liver Mitochondria

I. ACTIVITIES AT DIFFERENT pH VALUES

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Interest in the hydrolysis of adenosine triphosphate (ATP) by mitochondria was stimulated by the suggestion that it may be due to a reversal and diversion of the reactions which are responsible for the synthesis of ATP during the process of oxidative phosphorylation (Hunter, 1951; Lardy & Wellman, 1953; cf. Lardy & Elvehjem, 1945). When the mitochondria are carefully isolated from liver homogenates in isotonic sucrose solution at 0°, they exhibit little or no hydrolytic activity towards ATP (Kielley & Kielley, 1951). The oxidation of a number of substrates by these mitochondria is obligatorily coupled with the phosphorylation of two or three molecules of adenosine diphosphate (ADP) to ATP for each atom of oxygen consumed. The P:O ratio is decreased and the rate of hydrolysis of added ATP is increased when the structure of the mitochondria is damaged, e.g. by exposure to hypotonic solutions,

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incubations at 25–35° in the absence of substrate or exogenous ATP, addition of Ca^{2+} ions or surface-active agents, and physical disintegration in a Waring Blendor or by freezing and thawing. After any of these treatments the oxidation of a substrate may proceed unimpaired, but the presence of ADP and inorganic phosphate is no longer required in order to obtain the maximum rate of uptake of oxygen. The enzyme responsible for the dephosphorylation of ATP by mitochondria is usually designated as a 'latent ATPase', although it is not considered to be a simple hydrolytic enzyme or even a single enzyme (cf. Lardy & Wellman, 1953; Potter, Siekevitz & Simonson, 1953).

2:4-Dinitrophenol (DNP) and analogous phenols also stimulate the latent ATPase activity of normal mitochondria (Hunter, 1951; Potter & Recknagel, 1951; Lardy & Wellman, 1953; Potter *et al.* 1953) and produce a simultaneous uncoupling of the oxidative phosphorylation (Loomis & Lipmann, 1948). These effects appear to be reversible and can occur without any visible alteration in the mitochondrial structure. Similar results have been reported with a variety of other agents which have been less extensively investigated (cf. Hunter, 1955). It seems probable therefore that the uncoupling of oxidative phosphorylation is in some way related to the activation of the latent ATPase activity and that an investigation of the mechanism of hydrolysis of ATP might yield further information concerning the mechanism of oxidative phosphorylation.

The synthesis of ATP probably occurs at three separate points in the respiratory chain during the process of oxidative phosphorylation (cf. Lehninger, 1954; Slater, 1956; Lardy, 1956; Chance & Williams, 1956). Since the uncoupling of the phosphorylation seems to occur at all three points in the presence of DNP, there is a possibility that three separate enzymes or coenzymes might be involved in the hydrolysis of ATP. On investigating the effect of pH on the ATPase activity of mitochondrial preparations, we found evidence of four separate optimum pH values which possibly represent four different enzyme systems. Each of these activities appeared to be relatively specific for ATP. However, the effect of DNP in activating the latent ATPase activity was restricted to three of these pH values. It is possible therefore that these three values represent the three enzyme systems which might be involved in the oxidative phosphorylation. Preliminary reports of these results have been presented (Myers & Tuynman, 1956; Myers & Slater, 1957*a*).

METHODS

Enzyme preparations. Mitochondria were isolated from liver by the general method of Schneider & Hogeboom (1950) as modified by Hollünger (1955) and Hogeboom (1955). Male Wistar rats weighing about 250 g. were stunned and decapitated; a portion of the liver was immediately removed and chilled in cold 0·25 M-sucrose. The liver was weighed, cut into small pieces and ground for about 30 sec. at low speed in a chilled Potter–Elvehjem homogenizer with 10 ml. of cold 0·25 M-sucrose/g. of liver. The homogenizer used in all of these preparations was fitted with a polytetrafluoroethylene (Teflon) pestle and was obtained commercially from the Arthur H. Thomas Co., Philadelphia, Pa., U.S.A. (cf. Potter, 1955). The liver homogenate was subsequently centrifuged for 5 min. at 800 *g* in a cooled centrifuge to remove nuclei, erythrocytes, intact liver cells and debris; approximately three-quarters of the supernatant fluid was decanted and centrifuged for 10 min. at 6000–7000 *g*. The precipitate of mitochondria was resuspended in sucrose solution with the aid of the Potter–Elvehjem homogenizer and centrifuged for 10 min. at about 18 000 *g*. The fluffy layer was removed from the firmly packed sediment of mitochondria by gently shaking with small amounts of sucrose solution, and the residual mitochondria were again resuspended in sucrose solution with the aid of the homogenizer. All operations were carried out at 0°. This procedure, which was designed to isolate intact mitochondria rather than to recover all of the mitochondria present in the liver, yielded about 20 mg. of mitochondrial protein/g. of liver.

The preparations of sarcosomal fragments (cf. Cleland & Slater, 1953) from horse heart were usually isolated by the standard procedure of Keilin & Hartree (1947), as described by Slater (1949), in which the fragments are precipitated from an extract of the heart muscle by acidification to pH 5·7. Other preparations were precipitated by high-speed centrifuging (Slater, 1949).

Determination of enzyme activities. The method used for determining ATPase activity was based on the results obtained by Lardy & Wellman (1953) and Potter *et al.* (1953). The initial experiments were carried out at pH 7·4 in an isotonic medium containing 0·075 M-KCl, 0·108 M-sucrose, 0·001 M-ethylenediaminetetra-acetate (EDTA) and 0·002 M-ATP, with or without 0·003 M-$MgCl_2$ and with or without 0·0001 M-DNP. The total volume was 1·5 ml. The concentration of ATP is the same as that used by Potter *et al.* (1953); the concentration of DNP is intermediate between that used by Lardy & Wellman (1953) and by Potter *et al.* (1953), and is usually sufficient to produce complete uncoupling of oxidative phosphorylation in the respiratory chain. Potassium chloride is required for maximal stimulation of the latent ATPase activity of normal mitochondria by DNP (Lardy & Wellman, 1953); although this salt does not affect the ATPase activity of aged mitochondria it was included in all experiments for comparative purposes. The concentration of $MgCl_2$ is equal to the molar concentration of ATP (cf. Kielley & Kielley, 1951, 1953; Potter *et al.* 1953) plus the molar concentration of EDTA.

The EDTA was added to remove Ca^{2+} or other contaminating metal ions and appeared to be essential in order to obtain a low ATPase activity, in the absence of DNP, with normal mitochondria. It was not established whether this was due to an action of EDTA on the mitochondria (e.g. by removal of contaminating metals) or was due to metal-ion contamination of our sucrose and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) and possibly the ATP. The first two gave a positive reaction for metal ions with

8-hydroxyquinoline (cf. Chappell & Perry, 1953) and a titrimetric assay (Debney, 1952), which was carried out by Mrs A. J. Haarbrink-Haitsma, showed that the 0·25M-sucrose solutions contained about $2·6 \times 10^{-5}$M-calcium. The other solutions seemed to be free of metal impurities. The inhibition of the respiration of mitochondria by high concentrations of ATP suggested that the ATP might also contain impurities which were firmly bound and therefore not detectable by the hydroxyquinoline method. A concentration of 10^{-4}M-EDTA decreased the ATPase activity, in the absence of DNP, to a low level (Table 1). A concentration of 10^{-3}M-EDTA was used in most experiments.

In the experiments at pH 7·4, described in Table 1, the ATP used as substrate was the only buffer present. Subsequent experiments were carried out at different pH values in the presence of 0·05M-tris buffer. The pH values between 4·5 and 9·5 were obtained by titrating 0·25M-tris with 0·25M-acetic acid. To obtain stable buffers at pH 10–11, the 0·25M-tris was first mixed with an equal volume of aq. 0·25M-NH_3 soln. and subsequently titrated with 0·25M-acetic acid. In the descriptions of the experiments, both types of buffer are referred to as tris buffer. A concentration of 0·05M-tris means that these stock buffer solutions were diluted fivefold in the final reaction mixture. This degree of dilution did not appreciably alter the pH, nor was this affected by the concentration of ATP used. The tris–HCl buffer used in the experiment described in Fig. 4 was prepared by titrating 0·2M-tris with 2N-HCl. The histidine–HCl buffer used in the same experiment was prepared by titrating 0·2M-histidine hydrochloride with 2N-NaOH. Both these buffers were also diluted fivefold in the final reaction mixture. The reaction mixture used in these experiments contained 0·075M-KCl, 0·05M-sucrose, 0·05M-buffer, 0·001M-$MgCl_2$, 0·0006M-EDTA and 0·002M-ATP. The EDTA was usually omitted in experiments with fully activated preparations of aged or fragmented mitochondria.

Unless otherwise stated in the text, the mitochondrial preparations were incubated in the reaction mixture for 15 min. at 20±1°. The reaction was stopped by the addition of an equal volume of 10% (w/v) trichloroacetic acid, the protein was centrifuged off and the inorganic phosphate in the supernatant fluid determined by the Fiske–Subbarow method as modified by Sumner (1944).

The concentration of the enzyme preparation was adjusted so that not more than 40% of the ATP was hydrolysed; the same concentration of enzyme was used at all pH values in each experiment. Under these conditions, the amount of phosphate liberated was proportional to the enzyme concentration (cf. Lardy & Wellman, 1953), except with normal mitochondria in the absence of DNP (see Results). The concentration of normal liver mitochondria in the reaction mixture was usually 0·1–0·25 mg. of protein/ml.; most of the experiments with fragments of heart sarcosomes or liver mitochondria were carried out with approx. 0·03–0·1 mg. of protein/ml.

The protein content of the enzyme preparations was determined by the biuret method of Gornall, Bardawill & David (1949), as applied to mitochondrial preparations by Cleland & Slater (1953). The spectrophotometric measurement of the diphosphopyridine nucleotide (DPN) in mitochondria is described by Holton (1955) and by Holton, Hülsmann, Myers & Slater (1957).

Reagents. The crystalline disodium salt of ATP from muscle was supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A. Fresh samples of this reagent appeared to be free of any measurable amounts of inorganic phosphate, adenosine monophosphate (AMP) or ADP. AMP was also obtained from Sigma Chemical Co. ADP was prepared from ATP as described by Slater (1953). Sucrose, KCl and most of the other chemicals were AnalaR reagents supplied by British Drug Houses Ltd., Poole, England. EDTA was also obtained from British Drug Houses Ltd.

Other buffers. The imidazole–HCl buffer was prepared by titrating 0·2M-imidazole with 2N-HCl. The 5:5′-diethylbarbiturate–NaOH buffer was prepared by titrating 0·2M-sodium barbital with 2N-HCl. These were diluted fivefold in the final reaction mixture.

RESULTS

ATPase of isolated liver mitochondria at pH 7·4

The mitochondria isolated from rat-liver homogenates in 0·25M-sucrose had only a small activity towards ATP in the absence of DNP, provided that the isotonic reaction mixture contained EDTA (Table 1). Addition of Mg^{2+} ions to the reaction mixture generally decreased the activity still further (cf. Potter *et al.* 1953; Swanson, 1956). The residual activity towards ATP at pH 7·4 in the presence of Mg^{2+} ions depended on the purity of the ATP. The experiments shown in Table 2 were carried out with an old sample of ATP which contained inorganic phosphate and presumably ADP in amounts equivalent to about 9% of the total molar concentration of ATP; here the amount of free phosphate in the reaction medium actually decreased during the incubation with the liver mitochondria (Table 2). This result, which is similar to that found in some of the experiments reported by Kielley & Kielley (1951), is probably due to the synthesis of ATP from the free phosphate and ADP during the oxidation of endogenous substrates present in the mitochondria. Subsequent experiments were carried out with fresh samples of ATP which did not contain measurable amounts of ADP

Table 1. *Effect of ethylenediaminetetra-acetate on the hydrolysis of adenosine triphosphate by liver mitochondria*

The reaction mixture contained 0·075M-KCl, 0·108M-sucrose and 0·002M-ATP, pH 7·4; various amounts of EDTA, neutralized (pH 7·4) with NaOH, were also added. The mixture was incubated for 15 min. at 20° after the addition of liver mitochondria (0·073 mg. of protein/ml.).

EDTA (M)	μmoles of P/mg. of protein/hr.	
	No DNP	10^{-4}M-DNP
0	4·4	13·2
0·00001	2·7	12·5
0·0001	0·9	11·2
0·001	0·8	10·4
0·01	0·7	10·4

Table 2. *ATPase activity of liver mitochondria after isolation in various media*

Results presented here were obtained in a series of comparative experiments which were carried out during a period of 5 weeks in 1955. Reaction mixture contained 0·075 M-KCl, 0·108 M-sucrose, 0·001 M-EDTA and 0·002 M-ATP, with or without 0·003 M-$MgCl_2$ and with or without 0·0001 M-DNP. The mixture was incubated for 15 min. at 20° and pH 7·4 after addition of 0·2–0·4 mg. of mitochondrial protein/ml. The ATP used for these particular experiments, in contrast with the other experiments reported in this paper, contained appreciable amounts of inorganic phosphate and ADP (see text).

Isolation medium	No. of experiments	Average ATPase activity (μmoles of P/mg. of protein/hr.) No addition	DNP	$MgCl_2$	$MgCl_2$ + DNP
0·25 M-Sucrose	7	0·31	10·9	−0·27	10·0
0·25 M-Sucrose + 0·01 M-tris, pH 7·4	2	−0·13	11·4	+0·13	10·7
0·25 M-Sucrose + 0·01 M-tris, pH 6	1	0·0	—	0·0	8·4
0·25 M-Sucrose + 0·01 M-EDTA, pH 7·4	3	0·77	7·9	2·2	9·4
0·25 M-Sucrose + 0·01 M-EDTA + 0·01 M-$MgCl_2$, pH 7·4	1	0·0	11·8	1·8	11·7

Table 3. *Effect of the concentration of mitochondria on their ATPase activity*

The reaction medium used in the first series of determinations was the same as for Table 2 except that the ATP sample was free of inorganic phosphate and ADP. In the second series of determinations with the same mitochondria, the mixture of 0·075 M-KCl and 0·108 M-sucrose was replaced by 0·233 M-sucrose. The total amount of phosphate liberated by the complete hydrolysis of ATP to ADP should be equal to 2·8 μmoles. The small excess in the presence of Mg^{2+} ions and DNP is probably due to the formation of new ATP by the action of mitochondrial myokinase.

Mitochondria (mg. of protein/ml.)	KCl (M)	μmoles of P/15 min. No addition (*a*)	DNP (*b*)	$MgCl_2$ (*c*)	$MgCl_2$ + DNP (*d*)	(*b*/*a*)	(*d*/*c*)
1·48	0·075	0·15	2·90	0·06	3·55	19·4	59·3
0·74	0·075	0·22	2·78	0·08	2·81	12·6	35·1
0·37	0·075	0·22	2·13	0·08	1·84	9·7	23·0
0·148	0·075	0·14	0·87	0·05	0·74	6·2	14·8
0·074	0·075	0·07	0·44	0·025	0·37	6·3	14·8
1·48	0	0·40	2·42	0·19	2·36	6·0	12·4
0·74	0	0·38	2·13	0·21	1·98	5·6	9·4
0·37	0	0·27	1·48	0·21	1·05	5·5	5·0
0·148	0	0·16	0·55	0·15	0·43	3·4	2·8
0·074	0	0·08	0·28	0·07	0·21	3·5	3·0

Table 4. *Effect of the temperature on the ATPase activity of liver mitochondria*

Incubation temp.	μmoles of P/mg. of protein/hr. $MgCl_2$ (*a*)	$MgCl_2$ + DNP (*b*)	(*b*/*a*)
28°	2·3	28·5	12·4
21	1·3	16·1	12·4
14	0·4	5·8	14·5
1	0·0	0·4	—

or free phosphate. The incubation of the mitochondria with this preparation always resulted in the liberation of a small amount of inorganic phosphate under the same experimental conditions.

The addition of DNP to the reaction mixture increased the activity of the normal mitochondria at pH 7·4 in the presence of Mg^{2+} ions by 10–30 times. Similar increases were reported by Potter & Recknagel (1951), Lardy & Wellman (1953), Robertson & Boyer (1955), Witter, Watson & Cottone (1955) and Swanson (1956). The absolute rates of hydrolysis of ATP in the presence and absence of DNP were also similar to the rates reported by these investigators. As shown by Lardy & Wellman (1953), the presence of KCl was essential for maximal activity in the presence of DNP (Table 3).

The degree of stimulation of the latent ATPase activity was not markedly affected by the temperature of the reaction mixture (Table 4). However, it was highly dependent on the concentration of mitochondria (Table 3). Under our experimental conditions, the amount of phosphate liberated was proportional to the enzyme concentration only when the amount of mitochondrial protein did not exceed 0·5 mg./ml. In the absence of DNP, the amount of phosphate liberated actually decreased when much larger concentrations of mitochondria were used. The specific activity in the absence of DNP (μmoles of P/mg. of protein/hr.) thus decreased sharply from 2·52, with the two lower concentrations of mitochondria, to 0·27, with 1·48 mg. of protein/ml.

The pH of the liver homogenates and mitochondrial suspensions during the normal isolation procedure was in the range 6·5–6·7, regardless of the initial pH of the unbuffered sucrose isolation medium. The ATPase activities, measured under the four conditions in Table 2, were not appreciably affected by the addition of tris buffer to the sucrose solution in order to maintain the pH at 7·4 or at 6·0.

Addition of EDTA to the sucrose solution resulted in mitochondrial preparations with a moderately high ATPase activity in the absence of DNP (Table 2). This may be correlated with the observation that a distinct separation between the mitochondria and the fluffy layer was not obtained after the high-speed centrifuging in the EDTA–sucrose medium and with the fact that the addition of EDTA alters the structure of the microsomal fraction (Palade & Siekevitz, 1956). The ATPase activities could not be restored to normal by subsequently washing the mitochondria with 0·25 M-sucrose.

On the other hand, the EDTA did not seem to exert any deleterious effect on the normal mitochondria after they had been isolated in the usual way in sucrose solution; the mitochondria retained a low activity towards ATP when they were stored for 3–4 hr. at 0° either in sucrose or in sucrose solution containing EDTA. Moreover, normal mitochondria were more stable at 30° when EDTA was added to the suspension (Table 5). In the absence of EDTA, the ATPase activity was increased by pre-incubation for 2 hr. at 30° in sucrose solution (Kielley & Kielley, 1951; Lardy & Wellman, 1953; Potter *et al.* 1953). They also lose the ability to oxidize β-hydroxybutyrate (Table 5); a direct assay showed that the total amount of DPN in the mitochondria was decreased from 4·66 to 0·34 μ-mole/g. of protein, by incubation for 2 hr. at 30°. Both the inactivation of the β-hydroxybutyrate oxidase system and the activation of the ATPase were hastened by the addition of small amounts of Ca^{2+} ions (cf. Potter *et al.* 1953) and delayed by the addition of EDTA (Table 5). Similar results have been observed with heart sarcosomes (Slater & Cleland, 1953).

pH–*activity curves*

The pH–activity curves obtained with normal liver mitochondria in various reaction mixtures are shown in Fig. 1. The effect of EDTA in reducing the activity, at pH 7·4, in the absence of DNP is readily apparent. It had little effect in the presence of DNP. Addition of a small excess of Mg^{2+} ions did not significantly alter the curve obtained in the presence of DNP but decreased the small activity in the absence of DNP to still lower levels. Probably

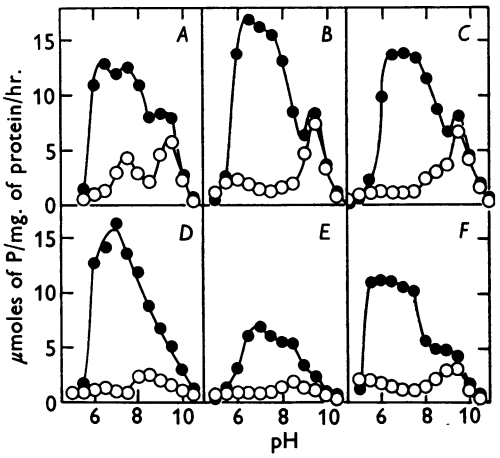

Fig. 1. Rate of hydrolysis of ATP by normal liver mitochondria at various pH values, in the absence (○) and the presence (●) of 10^{-4} M-DNP. The reaction mixture contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP and other components as follows: *A*, one experiment with no further additions; *B*, the average of three experiments with 0·0006 M-EDTA added; *C*, the average of twelve experiments with 0·0006 M-EDTA + 0·001 M-$MgCl_2$; *D*, one experiment with 0·0006 M-EDTA + 0·005 M-$MgCl_2$; *E*, one experiment with 0·0006 M-EDTA + 0·010 M-$MgCl_2$; *F*, one experiment with 0·01 M-EDTA.

Table 5. *Effect of pre-incubation at* 28° *on the enzymic activities of liver mitochondria*

A suspension of normal liver mitochondria in 0·25 M-sucrose with or without added EDTA or $CaCl_2$ was pre-incubated for different periods of time at 28°. Subsequently the ATPase activity was determined in the usual manner and the rate of oxidation of β-hydroxybutyrate was measured manometrically, in the medium of Copenhaver & Lardy (1952) with 0·001 M-EDTA added.

Pre-incubation at 28°		Oxidation of β-hydroxybutyrate	ATPase activity (μmoles of P/mg. of protein/hr.)	
Medium	Time (hr.)	(μl. of O_2/mg. of protein/hr.)	$MgCl_2$	$MgCl_2$ + DNP
0·25 M-Sucrose	0·0	39·5	−0·14	11·7
0·25 M-Sucrose	0·5	20·4	9·0	15·9
0·25 M-Sucrose	2·0	1·1	12·8	—
0·25 M-Sucrose + 0·002 M-EDTA	0·5	34·4	3·6	10·0
0·25 M-Sucrose + 0·002 M-$CaCl_2$	0·5	2·6	10·2	11·0

the presence of Mg^{2+} ions helps to maintain the normal mitochondrial structure during the incubation period of 15 min. at 20° (cf. Perry, 1956; Baltscheffsky, 1956). Higher concentrations of Mg^{2+} ions (0·005 M), approaching the values used in most experiments on oxidative phosphorylation, inhibit the ATPase activity at the higher pH values, and still higher concentrations inhibit the activity over the whole range of pH values (Fig. 1). Low concentrations of Mg^{2+} ions were used in subsequent experiments.

While these experiments were in progress, the effect of the pH on the hydrolysis of ATP by normal liver mitochondria in the absence of DNP was described by Swanson (1956). She also found a low ATPase activity at pH 7·0–7·5 but a much higher ATPase activity both at lower and at higher pH values. Under the conditions used in the present investigations normal liver mitochondria hydrolyse ATP slowly at all pH values between 4·5 and 8·5; a high ATPase activity is observed only in the region of pH 9·4–9·5 (Figs. 1, 2).

A comparison of the effects of different concentrations of DNP showed that 10^{-5} M-DNP produces a partial stimulation of the latent ATPase activity, which is greatest at about pH 6·5 (Fig. 2). A concentration which uncouples oxidative phosphorylation completely (10^{-4} M-DNP) produces no stimulation of the ATPase activity below pH 5, a maximal stimulation at pH 6·5, a partial stimulation in the region of pH 8·5 and no significant stimulation above pH 9·5 (Figs. 1, 2). When the concentration of DNP was increased to 10^{-3} M, the degree of stimulation at pH 6·5 was decreased, whereas at pH 8·5 it was increased (Fig. 2). Apparently, high concentrations of DNP inhibit the ATPase activity of normal mitochondria at pH 6·5; on the other hand, DNP did not exert any marked inhibitory effect on the ATPase activity after the enzymes had been activated by fragmentation of the mitochondria by freezing and thawing (Fig. 3).

Factors affecting the shape of the pH–*activity curves.* The effect of three different buffer systems is illustrated in Fig. 4. The results obtained with tris–HCl and histidine–HCl buffers were practically identical with those obtained with the usual tris

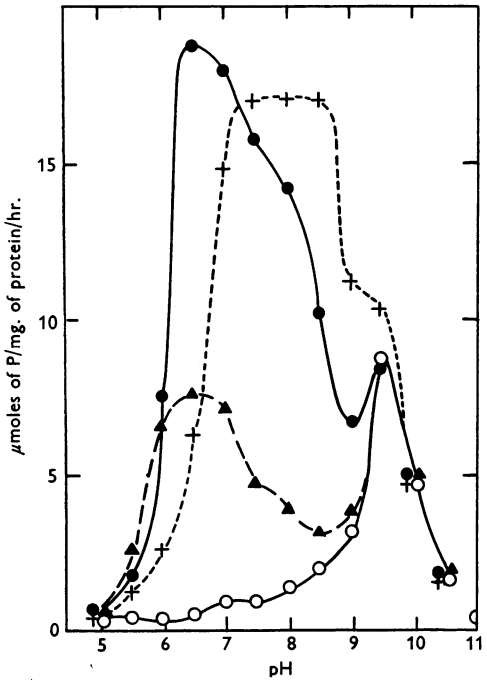

Fig. 2. Effect of different concentrations of DNP on the rate of hydrolysis of ATP by normal liver mitochondria. The reaction mixture contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP, 0·001 M-$MgCl_2$ and 0·0006 M-EDTA. The following concentrations of DNP were added: ○, none; ▲, 10^{-5} M; ●, 10^{-4} M; +, 10^{-3} M.

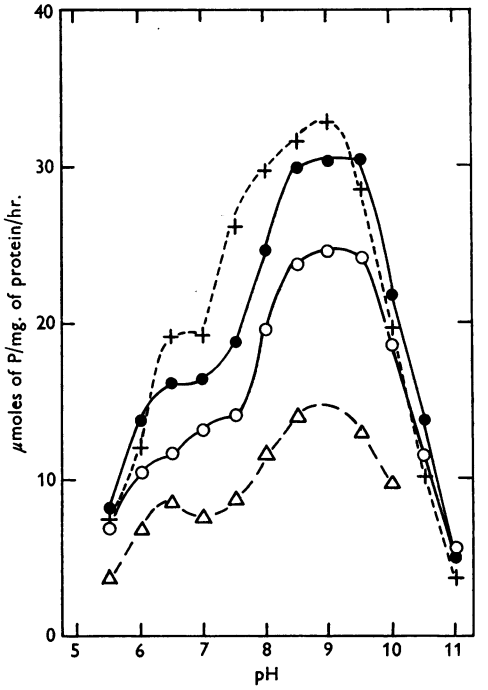

Fig. 3. Effect of DNP on the rate of hydrolysis of ATP by preparations of fragmented liver mitochondria. The reaction mixture was the same as given for Fig. 2. ATPase activities of the mitochondria were measured in the absence of DNP after the mitochondrial preparations had been subjected to freezing and subsequent thawing once (△) and twice (○). Activities of the latter preparation were also measured in the presence of 10^{-4} M-DNP (●) and of 10^{-3} M-DNP (+).

buffer (containing acetic acid and, at the higher pH values, aq. NH_3, see p. 560). 5:5-Diethylbarbiturate–NaOH and imidazole–HCl buffers appeared to be less satisfactory, since the maximum rate of hydrolysis of ATP was decreased appreciably; however, the shape of the pH–activity curves was not altered greatly. A few experiments were also carried out with no buffer apart from the substrate. The ATP had little buffer capacity in the reaction mixture above pH 8; however, the results obtained up to this point were similar to those obtained in the presence of tris buffer. It seems probable therefore that the pH–activity curves are not distorted in any way by the presence of tris, acetate and (at the higher pH) NH_4^+ ions in the reaction mixtures.

The effect of the concentration of Mg^{2+} ions on the ATPase activity of normal mitochondria was mentioned above (cf. Fig. 1). This was studied in more detail with a fully activated preparation of aged mitochondria which had been subjected to repeated freezing and thawing (Fig. 5). Normal mitochondria contain appreciable amounts of bound magnesium (Siekevitz & Potter, 1955) and do not require added Mg^{2+} ions to be able to hydrolyse ATP at a maximal rate in the presence of DNP (cf. Fig. 1) (Lardy & Wellman, 1953; Potter *et al.* 1953). However, after ageing, followed by freezing and thawing, the mitochondrial suspensions have little ATPase activity in the absence of added Mg^{2+} ions (Fig. 5). The pH–activity curve obtained with our preparation of frozen mitochondria in the presence of Mg^{2+} ions is similar to that given by Kielley & Kielley (1953) for their purified preparation of mitochondrial fragments. The effect of the concentration of Mg^{2+} ions is also similar to that reported by Kielley & Kielley (1953), when the differences in substrate concentration are taken into consideration; as shown by Kielley & Kielley (1953), the optimum concentration of Mg^{2+} ions is directly proportional to the concentration of ATP. The maximum activity in our experiments was produced

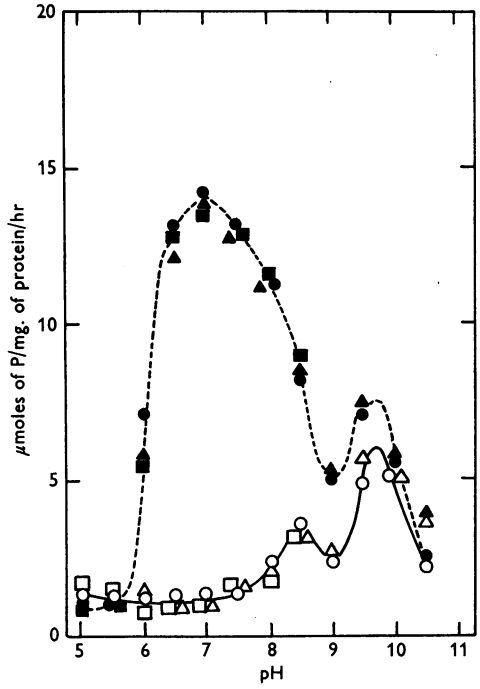

Fig. 4. Effect of different buffers on the hydrolysis of ATP by normal mitochondria. The reaction mixture was the same as that given for Fig. 2 with the exception of the buffer system. The ATPase activities of a normal mitochondrial preparation were measured in the presence of the usual tris buffer (0·05 M), with (●) and without (○) 10^{-4} M-DNP; in the presence of tris–HCl buffer with (▲) and without (△) 10^{-4} M-DNP, and in the presence of histidine–HCl buffer, with (■) and without (□) 10^{-4} M-DNP. For the composition of these buffers see Methods, p. 560.

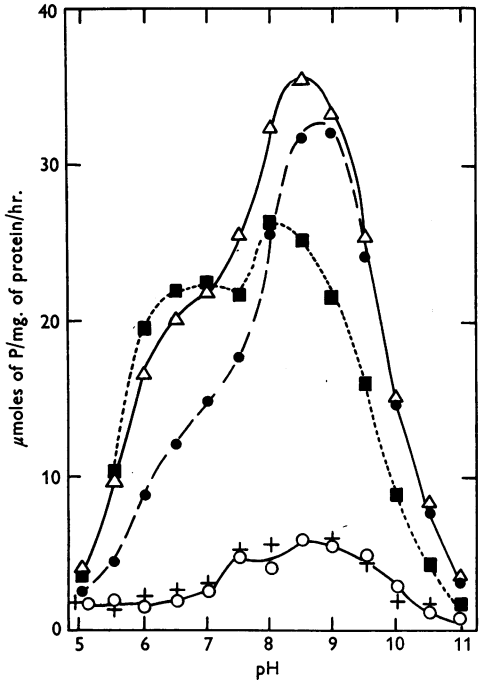

Fig. 5. Effect of different concentrations of $MgCl_2$ on the hydrolysis of ATP by a preparation of liver mitochondria, after ageing, followed by freezing and thawing. The reaction mixture was the same as that given for Fig. 1*A*. Liver mitochondria were aged by incubation for 2 hr. at 30° in 0·25 M-sucrose and subsequently frozen and thawed five times in succession. ATPase activities were measured with no added $MgCl_2$ (○) and with 0·0003 M- (●), 0·001 M- (△) and 0·003 M-$MgCl_2$ (■) added. The effect of 0·001 M-$CaCl_2$ (+) was also determined.

with a concentration of Mg^{2+} ions equal to one-half of the molar concentration of ATP. Concentrations of Mg^{2+} ions equal to 1·5 times the molar concentration of ATP caused inhibition above pH 7·5 (Fig. 5). When the Mg^{2+} concentration equalled five times that of the ATP, the ATPase was inhibited at all pH values between 6 and 10; a similar result was observed with normal mitochondria (Fig. 1). The differences in the optimum concentrations of Mg^{2+} ions at different pH values probably reflect the effect of the pH on the dissociation of ATP and its affinity for Mg^{2+} ions (Martell & Schwarzenbach, 1956; Nanninga, 1956). The reason for the inhibitory effect of excess of Mg^{2+} ions is still obscure but it is possible that ATP itself rather than a magnesium complex of ATP serves as the substrate for an enzyme which is only active when combined with magnesium. The effect of the concentration of Mg^{2+} ions on the ATPase activity of heart sarcosomes (isolated according to the procedure of Cleland & Slater, 1953) and sarcosomal fragments was found to be similar to the effects described above. A concentration of 0·001 M-Mg^{2+} ions in the presence of 0·002 M-ATP seemed most suitable for subsequent experiments.

Calcium ions do not stimulate the hydrolysis of ATP by aged mitochondria at pH 7·4 (Lardy & Wellman, 1953), even though calcium is also firmly bound by ATP (Martell & Schwarzenbach, 1956). None of the ATPase activities between pH 5 and 11 was in fact activated by Ca^{2+} ions (Fig. 5); thus these enzymes differ in this respect from the ATPase of myosin (cf. Engelhardt, 1946). On the other hand, the hydrolysis of ATP was activated by Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+} (Fig. 6) and by Sn^{2+} as well as by Mg^{2+} ions. Similar experiments with sarcosomal fragments have shown that Cd^{2+}, Ni^{2+} and Be^{2+} ions also produce a considerable activation; Al^{3+}, Ba^{2+} and Cu^{2+} ions were ineffective or inhibitory. These experiments were carried out at pH 7·4 in the same way as the experiments shown in Fig. 6; other experiments showed that 3×10^{-4} M-$MnCl_2$ and $SnCl_2$ were equally effective over the whole pH range where the salts remained soluble. Activation of the ATP hydrolysis by Mn^{2+} ions has been reported previously for other enzyme preparations which are activated by Mg^{2+} ions (Kielley & Meyerhof, 1948; Meyerhof & Ohlmeyer, 1952; Sacktor, 1953); apparently a large number of bivalent cations are almost equally effective. There was no

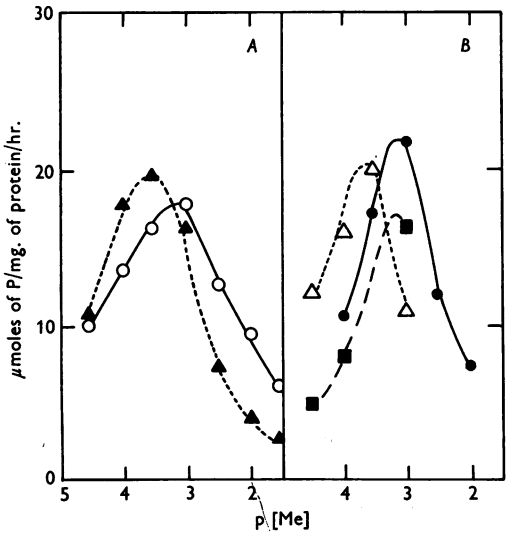

Fig. 6. (*A*, *B*). Effect of different ions on the hydrolysis of ATP by aged liver mitochondria at pH 7·4. The reaction mixture contained 0·075 M-KCl, 0·108 M-sucrose and 0·002 M-ATP, pH 7·4. The liver mitochondria were aged by incubation for 2 hr. at 30° in 0·25 M-sucrose solution and their ATPase activities measured in the presence of various concentrations of $MgCl_2$ (○), $MnSO_4$ (▲), $CoCl_2$ (●), $ZnSO_4$ (△) and $FeSO_4$ (■). ATPase activity is plotted against p[Me], the negative logarithm of the concentration of the ions (g. ions/l.). Similar results were obtained with $ZnSO_4$ and $ZnCl_2$.

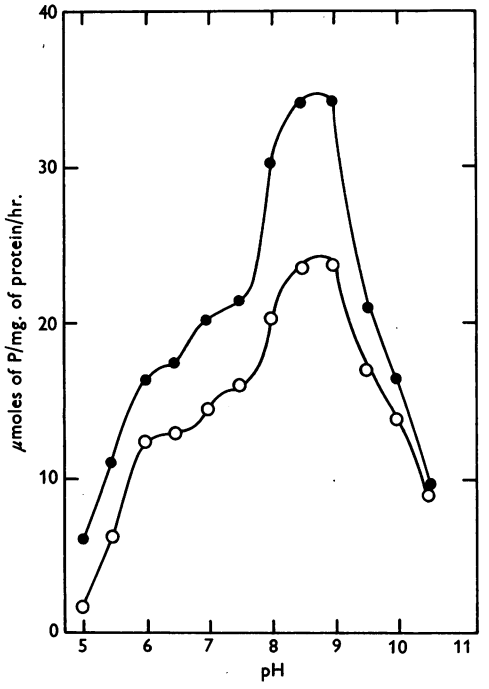

Fig. 7. Effect of the concentration of ATP on the hydrolysis of ATP. Liver mitochondria were aged by incubation for 2 hr. at 30° and subsequently frozen and thawed. ATPase activities were measured in the presence of 0·002 M-ATP + 0·001 M-$MgCl_2$ (○) and of 0·01 M-ATP + 0·005 M-$MgCl_2$ (●) in a reaction medium containing 0·075 M-KCl, 0·05 M-sucrose and 0·05 M-tris buffer.

evidence of a slow reaction between the enzyme and the cation. Identical results were obtained when the mitochondrial preparations were added to a mixture containing Mg^{2+} ions and ATP or when they were pre-incubated with Mg^{2+} ions at 20° for between 15 min. and 3 hr., and the ATP was added later.

The absolute rate of hydrolysis of ATP by frozen mitochondria was increased by 30–50% when the concentration of ATP was increased fivefold (Fig. 7); however, the shape of the pH–activity curve was not altered markedly.

Substrate specificity

Although the enzyme responsible for the hydrolysis of ATP at pH 7·4 appears to be relatively specific for this substrate (Kielley & Kielley, 1953; Lardy & Wellman, 1953; Sacktor, 1953; cf. Perry, 1956), it seemed possible that some of the other optimum pH values might be due to the presence of other enzymes which are not specific for ATP. For example, the normal preparations of liver mitochondria are inevitably contaminated with a small number of lysosomes which contain an acid phosphatase capable of hydrolysing glycerol phosphate (Appelmans, Wattiaux & de Duve, 1955; de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). The presence of this enzyme in our mitochondrial preparations could be confirmed (Table 6); however, the rate of hydrolysis of glycerol phosphate is small in comparison with the rate of hydrolysis of ATP, and was not stimulated by DNP (cf. Appelmans & de Duve, 1955). Similar results were obtained with phenyl phosphate, AMP and inorganic pyrophosphate as substrates (Table 6). It seems improbable that an acid phosphatase, alkaline phosphatase or pyrophosphatase could be responsible for a significant portion of the DNP-stimulated ATPase activity of normal mitochondria.

The hydrolysis of ADP by normal mitochondria was stimulated by the addition of DNP (Table 6), but this can be explained by the combined action of adenylkinase and a DNP-stimulated ATPase.

Table 6. *Hydrolysis of different phosphate esters by normal liver mitochondria*

The reaction medium contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·001 M-$MgCl_2$, 0·0006 M-EDTA and 0·002 M-phosphate ester, with or without 10^{-4} M-DNP. The mixture was incubated for 20 min. at 20° after the addition of 0·18 mg. of mitochondrial protein/ml. The liberation of inorganic phosphate was less than 0·05 μmole/mg. of protein/hr. in all cases where the value is omitted. Parallel experiments showed a similar rate of hydrolysis of the inorganic pyrophosphate and other esters by a preparation of aged and frozen mitochondria, although ATP is hydrolysed more rapidly by these preparations (Figs. 3, 5 and 8).

	Rate of enzymic hydrolysis (μmoles of P/mg. of protein/hr.)					
	ATP		ADP		AMP	
pH	$MgCl_2$	$MgCl_2$ + DNP	$MgCl_2$	$MgCl_2$ + DNP	$MgCl_2$	$MgCl_2$ + DNP
5·0	1·2	1·2	0·2	0·1	0·1	0·1
5·5	1·7	2·6	0·4	0·8	0·2	0·2
6·0	1·4	11·2	0·3	1·4	0·4	0·4
6·5	1·2	12·2	0·2	1·3	0·2	0·2
7·0	1·1	12·2	0·1	1·2	0·2	0·2
7·5	1·2	12·1	0·2	1·1	0·2	0·2
8·0	3·0	10·5	0·7	1·1	0·2	0·2
8·5	3·4	7·8	0·9	1·1	0·2	0·1
9·0	5·1	8·8	0·9	1·1	0·2	0·1
9·5	7·1	9·9	0·9	1·1	0·3	0·1
10·0	5·3	5·7	0·6	0·7	0·2	0·1
10·5	2·5	2·5	0·5	0·5	0·1	0·1
	β-Glyceryl phosphate		Phenyl phosphate		Inorganic pyrophosphate	
	$MgCl_2$	$MgCl_2$ + DNP	$MgCl_2$	$MgCl_2$ + DNP	$MgCl_2$	$MgCl_2$ + DNP
5·0	0·1	0·1	0·4	0·4	0·8	0·2
5·5	0·1	0·2	0·3	0·3	0·4	0·1
6·0	0·2	0·2	0·4	0·4	0·2	0·1
6·5	0·1	0·1	0·1	0·1	0·1	—
7·0	—	—	—	—	0·1	0·1
7·5	—	—	—	—	0·3	0·3
8·0	—	—	—	—	0·2	0·1
8·5	—	—	—	—	0·1	—
9·0	0·1	—	—	—	0·4	—
9·5	—	—	—	—	0·8	—
10·0	—	0·0	—	—	0·3	—
10·5	0·0	0·0	—	—	—	—

ATPase activity at pH 9·4

In contrast with the results obtained at lower pH values, the ATPase activity of normal mitochondria at pH 9·4 is relatively high in the absence of DNP and is scarcely affected by the addition of DNP (Figs. 1–3). It was necessary to consider the possibility that the rapid hydrolysis of ATP by normal mitochondria at pH 9·4 might reflect the effect of the alkaline pH on the structure of the mitochondria. The mitochondria suspensions in a series of tubes containing buffers at different pH values were all equally turbid initially; however, the suspensions gradually became translucent in the buffers above pH 9 during the course of the incubation period of 15 min. at 20°, and no change was observed at lower pH values. However, the slow increase in translucency at pH 9·5 was not associated with an increased ATPase activity, since the amount of ATP hydrolysed at pH 9·5 increased linearly with the time of incubation (Table 7). In the same experiment, concentrated suspensions of the mitochondria were also pre-incubated for 15 min. in the same reaction mixtures at pH 6·0, 7·5 and 9·5; each suspension was subsequently diluted in a series of buffers for determination of the ATPase activity in the usual way. If the mitochondria were seriously damaged by the pre-incubation at pH 9·5, it would be expected that the activity at lower pH values in the absence of DNP should also be increased by this period of pre-incubation. The increase in the activity at lower pH values proved to be relatively small (Table 7). Further evidence will be presented below which suggests that the high ATPase activity at pH 9·4 is due to a separate enzyme system (cf. Figs. 8, 9).

Effect of ageing and fragmentation of the mitochondria. The effect of ageing on the mitochondria has been described by various authors (Kielley & Kielley, 1951; Lardy & Wellman, 1953; Potter *et al.* 1953). After pre-incubation of the mitochondria

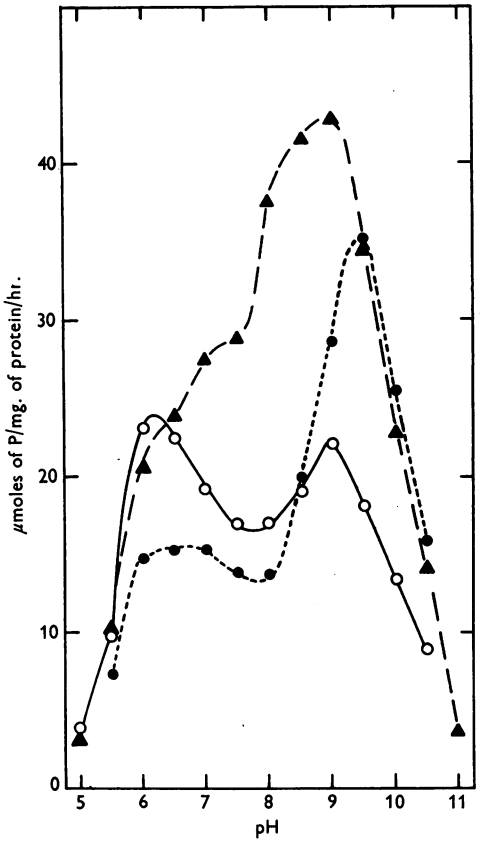

Fig. 8. Rate of hydrolysis of ATP by a preparation of liver mitochondria after subjection to various treatments. The reaction mixture contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP and 0·001 M-$MgCl_2$. A preparation of normal liver mitochondria was aged by incubation for 2 hr. at 30° in 0·25 M-sucrose (○); subsequently a portion of this aged preparation was stored for 1 day at 2° (●), and another portion was stored for 1 day at −18° (▲).

Table 7. *Effect of the incubation period on the latent ATPase activities of normal liver mitochondria*

The reaction medium was the same as for Table 6, with 0·002 M-ATP as substrate and without DNP. In the first series of measurements with no pre-incubation of the mitochondria, the mixtures were incubated at 20° for different periods of time after the addition of 0·25 mg. of mitochondrial protein/ml. In the second series of experiments, a more concentrated suspension of the same preparation of mitochondria (3·75 mg. of protein/ml.) was incubated for 15 min. at 20° in three reaction mixtures of the same composition as was normally used for the ATPase assay; subsequently small samples of these concentrated suspensions were diluted 15-fold in a series of tubes containing the reaction mixtures and the ATPase assay was carried out in the usual manner.

pH during pre-incubation	Incubation period for ATPase assay (min.)	ATPase activity (μmoles of P/mg. of protein/hr.) at pH 5·5–10·0									
		5·5	6·0	6·5	7·0	7·5	8·0	8·5	9·0	9·5	10·0
—	5	1·0	1·3	1·8	1·5	1·6	2·2	2·6	3·2	4·5	3·2
—	15	0·7	0·7	1·0	0·8	1·1	1·7	2·2	2·4	4·8	3·3
—	30	0·7	0·7	1·0	0·8	1·0	1·7	2·2	2·4	4·8	3·2
6·0	15	1·0	0·9	1·3	1·2	1·5	2·8	3·0	3·2	4·5	3·2
7·5	15	1·0	0·8	1·2	1·0	1·4	2·3	2·5	—	4·0	3·2
9·5	15	1·0	1·5	1·9	1·8	2·0	2·6	2·7	3·0	4·5	2·4

suspensions in sucrose solution for 2 hr. at 30°, the rate of hydrolysis of ATP at pH 7·4 in the presence of Mg^{2+} ions reached a value which was consistently a little higher than the maximum observed with normal mitochondria in the presence of DNP (Fig. 8; cf. Potter *et al.* 1953). Considerably higher values were observed at pH 6·3 and 9; the position of the maximum at pH 9 is probably due to the superimposition of two pH–activity curves with maxima near pH 8·5 and 9·4. However, the ATPases are still not fully activated by ageing; the activities at pH 7·4, 8·5 and 9·4 increased greatly when the preparation of aged mitochondria was subsequently frozen and thawed. On the other hand, the ATPase activity at pH 6·3 appeared to have reached its maximum after ageing and was scarcely affected by the freezing procedure. When the suspension of aged mitochondria was stored at 2° for 1 day, the activity at pH 6·3 was considerably reduced, whereas that at pH 9·4 increased to its maximal value. The results obtained in another experiment suggest that similar changes may occur when the aged mitochondria are incubated for a further 3 hr. at 30°. These data show that different degrees of activation of the ATPase activities at different pH values can be achieved by subjecting the mitochondria to various treatments.

The relative heights of the maxima at pH 6·3 and 9 varied considerably from one preparation of aged mitochondria to another. On the other hand, the results obtained with normal mitochondria (Fig. 1) and with the fully activated preparation of aged frozen mitochondria (Fig. 8) were consistently reproducible. The variability in the pH–activity curves obtained with aged mitochondria is probably due to differences in the uncontrolled factors, e.g. the calcium content, pH and concentration of mitochondria in the suspension during the incubation at 30°. In one experiment, two portions of the same suspension of mitochondria were aged separately under aerobic and anaerobic conditions (cf. Hunter, Davis & Corlat, 1956); the results obtained with these two preparations were practically identical.

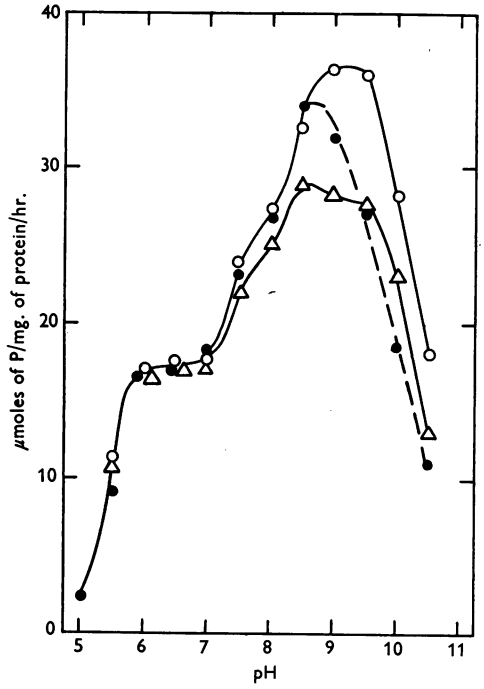

Fig. 9. Hydrolysis of ATP by a preparation of normal liver mitochondria which had been subjected to freezing and thawing three (△), five (○) and six (●) times in succession. The reaction medium is the same as that given for Fig. 8.

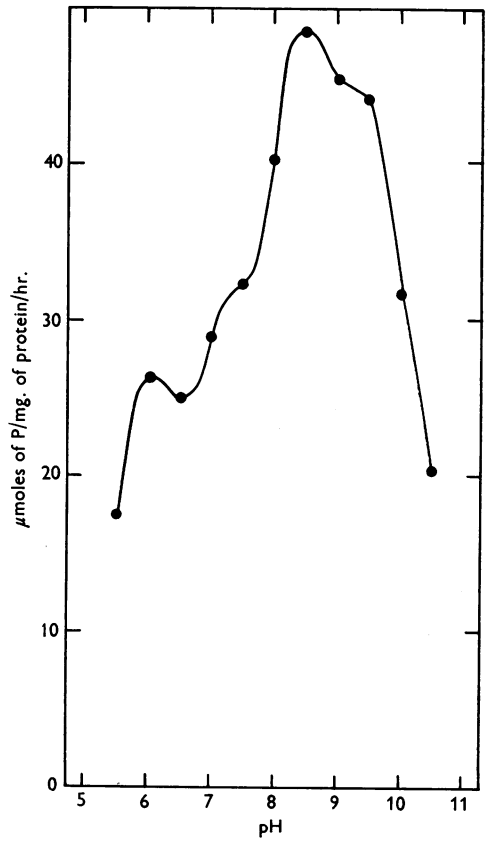

Fig. 10. Hydrolysis of ATP by mitochondrial fragments. A preparation of normal liver mitochondria was suspended in cold 0·003 M-tris buffer, pH 7·5, and homogenized in a Waring Blendor for 10 min. Intact mitochondria and inactivated particles were centrifuged off at 18 000 *g* and the ATPase activities of the mitochondrial fragments in the supernatant fluid were measured in the same reaction medium as given for Fig. 8.

The effect of freezing and thawing is probably due to fragmentation of the mitochondria. A single freezing is sufficient to produce a maximal or near-maximal activation of the ATPase of aged mitochondria. However, the maximal activity was still not attained after freezing and thawing unaged mitochondria five or six times (cf. Figs. 3 and 9). Repeated freezing and thawing of an aged or a normal preparation of mitochondria was sometimes accompanied by a precipitation of some of the protein material and a loss of the ATPase activity above pH 8·5 (Fig. 9).

Activation of the ATPase was also brought about by suspension of normal mitochondria in a hypotonic medium at 0° and treatment in a Waring Blendor (Fig. 10), as described by Kielley & Kielley, 1953 (Fig. 10). However, this preparation was not stable at either −18° or 0° (cf. Hollünger, 1955). The preparations of frozen mitochondria in sucrose solution were also unstable when stored at 0° (Fig. 11), but could be stored in a frozen state at −18° for at least 3 months with little change in activity.

The ATPases present in a fully activated preparation of frozen aged mitochondria were all inactivated to a similar extent when this preparation was allowed to stand for 1 day at 0° in sucrose solutions at various pH values (Fig. 11). The stability of the enzymes appears to be maximal at pH 7–8 under these conditions. A similar result was obtained when the mitochondrial preparations were incubated at more extreme pH values for 30 min. at 20°. The enzymes can also be inactivated by exposure to temperatures above 40° for 30 min. at pH 7·5; again no evidence of a marked selectivity in the degree of inactivation was observed.

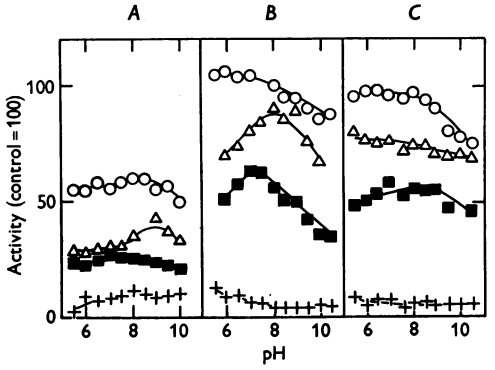

Fig. 11. Stability of the ATPase activities. The reaction medium was the same as that given for Fig. 8. (*A*) ATPase activities of mitochondria which had been frozen and thawed were measured after standing for 23 hr. at 0° in 0·2 M-sucrose + 0·05 M-tris buffer at pH 5 (+), pH 6 (△), pH 7 (○) and pH 9 (■). Similar results were obtained after standing at pH 7 and at pH 8. (*B*) ATPase activities were measured after the mitochondrial preparations were pre-incubated for 30 min. at 20° in 0·05 M-sucrose + tris buffer at pH 3·8 (+), pH 4·8 (△), pH 9·8 (○) and pH 11·5 (■). (*C*) ATPase activities were measured after the mitochondrial preparations were pre-incubated for 30 min. at pH 7·5 in 0·05 M-sucrose + 0·05 M-tris buffer at 35° (○), 45° (△), 50° (■) and 55° (+).

DISCUSSION

The pH–activity curves of the hydrolysis of ATP by liver mitochondria, treated in various ways to activate the latent ATPase, show evidence of four different peaks, at pH 6·3, 7·4, 8·5 and 9·4. The different curves often overlapped one another to give peaks at intermediate pH values, but it was possible under appropriate conditions to demonstrate each peak separately from the others.

The existence of four different peaks could conceivably be explained by one or a combination of the following factors:

(1) The effect of pH on the concentration of the ionic species of ATP (or of an ATP–magnesium complex) which is the substrate of the enzyme.

(2) The effect of pH on the permeability of the mitochondria to ATP.

(3) The presence in mitochondria of four different enzymes (or enzyme systems) which bring about the hydrolysis of ATP.

The first two factors would be expected to be important only at low concentrations of ATP. In our experiments, a high concentration of ATP (0·002 M) in the presence of an optimum concentration of Mg^{2+} ions (0·001 M) was used, and a fivefold increase in these concentrations increased the rate of hydrolysis of ATP only slightly, without altering the shape of the pH–activity curve. Moreover, neither of these factors would explain the differential effects of different concentrations of DNP. For these reasons we consider that the first two factors do not contribute to an important extent to the shape of the pH–activity curves, which we interpret in terms of the presence in mitochondria of four different enzyme systems bringing about the hydrolysis of ATP. These four enzymes will be designated by their optimum pH values, namely 6·3, 7·4, 8·5 and 9·4. This interpretation is strongly supported by our more recent work (Hülsmann & Slater, 1957) which shows that oxidative phosphorylation (as measured by the P:O ratio) in heart sarcosomes has three peaks (at pH 6·3, 7·4 and 8·5) with glutamate as substrate, two with succinate (at pH 7·4 and 8·5) and one with ascorbate (at pH 6·3). Under these conditions the permeability of the mitochondria to ATP cannot be a factor.

The four different enzyme systems are characterized by the following properties.

9·4 *enzyme*. This is the only enzyme which is appreciably active in freshly prepared liver

mitochondria, in the presence of EDTA and in the absence of uncoupling agents (Fig. 1 *B*). It was shown that this activity is not due to irreversible structural damage, brought about by the high pH. This enzyme is not stimulated by DNP (up to 10^{-3} M), but is markedly activated by ageing of the mitochondria, followed by freezing and thawing.

8·5 *enzyme*. This enzyme, like the 7·4 and 6·3 enzymes, is stimulated by DNP, but requires a higher concentration of this reagent for full activation. It is most clearly demonstrated by plotting the difference between the activity with 10^{-3} M-DNP and with 10^{-4} M-DNP (Fig. 12). A clear peak at this pH is also shown in mitochondria damaged in the Waring Blendor (Fig. 10), and in the effect of Mg^{2+} ions on the ATPase activity of heart sarcosomes (Holton *et al.* 1957).

7·4 *enzyme*. Although the peak of this enzyme is usually obscured by the presence of neighbouring peaks, an inflexion in this region is often obtained (e.g. Fig. 12). A clear peak was obtained at pH 7·4, when measurements were made in the absence of EDTA, either in the presence or in the absence of DNP or Mg^{2+} ions (Fig. 1 *A*; see also Myers & Slater, 1957*a* for another experiment in the presence of 0·001 M-$MgCl_2$). This enzyme is also greatly activated by sodium dithionite (Myers & Slater, 1957*b*).

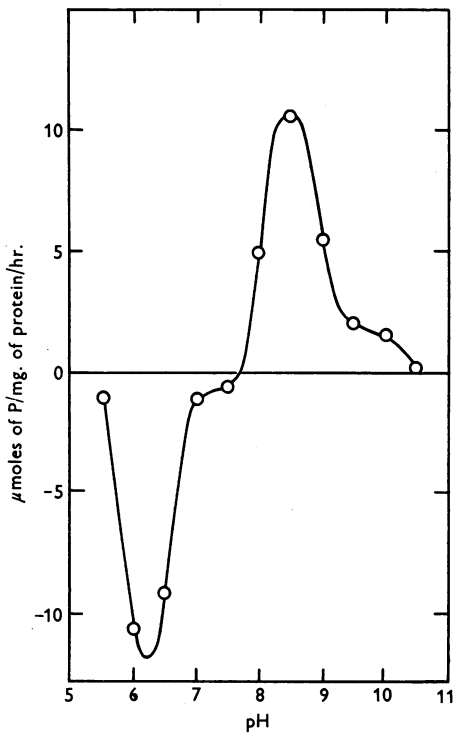

Fig. 12. Difference curve plotting the ATPase activity in the presence of 10^{-3} M-DNP minus the activity in the presence of 10^{-4} M-DNP. The reaction mixture contained 0·075 M-KCl, 0·05 M-sucrose, 0·05 M-tris buffer, 0·002 M-ATP and 0·001 M-EDTA (no added Mg^{2+} ions). Liver mitochondria, 0·06 mg. of protein/ml.

6·3 *enzyme*. This was the only enzyme activated by 10^{-5} M-DNP (Fig. 2) or by calcium or azide (Myers & Slater, 1957*b*). It is further activated by 10^{-4} M-DNP, but is inhibited by 10^{-3} M-DNP as is clearly seen in Fig. 12. A peak at this pH is also shown in curve 2 of Fig. 1 of Myers & Slater (1957*a*). This enzyme is also activated by incubation for 2 hr. at 30° (Fig. 8) and, unlike the other three enzymes, is not further activated by freezing and thawing the aged mitochondrial preparations.

The decreasing activity of DNP with increasing pH might at first suggest that the activator is the undissociated phenol, which decreases in concentration with increasing pH (cf. Brigham, Brinch-Johnsen & Walaas, 1956). However, this appears extremely unlikely since the p*K* values of DNP and *p*-nitrophenol are 4 and 7 respectively (Pauling, 1940), so that the concentration of the undissociated phenols in 10^{-4} M-solutions at pH 7·4 are 3×10^{-8} M and 3×10^{-5} M respectively. Nevertheless, *p*-nitrophenol and DNP are about equally effective in uncoupling oxidative phosphorylation. Thus it appears clear that the active agent in DNP is the ion and that changes of pH within the range studied affect the enzyme system rather than the inhibitor.

Liver mitochondria, activated by ageing followed by freezing and thawing, give a pH–activity curve which can also be interpreted in terms of the four enzymes. This curve is dominated by the enzymes in the alkaline region, and the main peak at about 8·8 can be considered to be a composite curve of the 8·5 and 9·4 enzymes. The 6·3 and 7·4 enzymes cause inflexions of the curve (Fig. 7).

Although the four different ATPases were activated to different extents by various means, their stabilities in the fully activated preparation to the treatments described in Fig. 11 were about the same. This could be because a factor common to all four enzyme systems is destroyed by these treatments. The marked inhibition by higher concentrations of Mg^{2+} ions at the higher pH values could be due to the effect of pH on the formation of an inactive magnesium–ATP complex, and does not necessarily represent a different degree of susceptibility of the various enzymes to Mg^{2+} ions.

The results of this investigation confirm earlier suggestions that the hydrolysis of ATP by mitochondrial preparations is not a simple process. The evidence that there are three DNP-sensitive ATPase systems is consistent with the postulate (Hunter, 1951; Lardy & Wellman, 1953) that these enzymes represent a reversal and diversion of the reactions which are responsible for the synthesis of ATP by

oxidative phosphorylation, and with the experimental evidence that there are three DNP-sensitive steps in oxidative phosphorylation. This postulate is given further strong support by our recent finding that oxidative phosphorylation has optimum P:O ratios at the same pH values as the DNP-stimulated ATPase and that the number of optimum values with different substrates is the same as the number of phosphorylating steps with these substrates (Hülsmann & Slater, 1957).

SUMMARY

1. Normal liver mitochondria hydrolyse adenosine triphosphate (ATP) slowly at all pH values between 4·5 and 8·5. The addition of an uncoupling agent such as 2:4-dinitrophenol produces a marked stimulation of the ATPase activity between pH 6 and 8·5. However, the maximal enzymic activity is only attained when the mitochondrial structure has been disintegrated, e.g. by repeated freezing and thawing.

2. The pH–activity curves obtained with mitochondrial fragments or with normal mitochondria in the presence of 2:4-dinitrophenol appear to represent four superimposed optimum pH values which are located in the regions of pH 6·3, 7·4, 8·5 and 9·4. This is interpreted in terms of the presence in mitochondria of four different ATPase systems. All of these hydrolytic enzymes seem to be relatively specific for ATP.

3. The 9·4 enzyme is the only one which is appreciably active in freshly prepared liver mitochondria, in the presence of ethylenediaminetetra-acetate and absence of uncoupling agents. This activity is not due to irreversible structural damage, brought about by the high pH. This enzyme is not stimulated by 2:4-dinitrophenol but is activated by ageing followed by freezing and thawing.

4. The 8·5 enzyme is stimulated by 2:4-dinitrophenol but requires a higher concentration than the 7·4 and 6·3 enzymes for full activation. It is also activated by damaging the mitochondria.

5. The 7·4 enzyme is stimulated by 2:4-dinitrophenol, by mechanical damage to the mitochondria and by removal of ethylenediaminetetra-acetate from the reaction medium.

6. The 6·3 enzyme is the only one clearly activated by 10^{-5} M-2:4-dinitrophenol, and is also fully activated by ageing the mitochondria.

7. The 6·3, 7·4 and 8·5 enzymes, which are stimulated by 2:4-dinitrophenol, may be related to the three steps in the respiratory chain which are coupled with phosphorylation.

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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.