LATENT ADENOSINETRIPHOSPHATASE ACTIVITY IN RESTING RAT LIVER MITOCHONDRIA*

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Both calcium and magnesium ions have been shown to activate the breakdown of ATP in whole homogenates of rat liver (2, 3), but purified ATPase from mitochondria was activated only by magnesium (4). Intact mitochondria have yielded divergent results (5, 6) with respect to the effect of calcium on their ATPase activity. These differences may depend on the state of the mitochondria, as will be shown in this paper.

Freshly isolated rat liver mitochondria have very little ATPase activity (7-9), but a great increase in activity results from the addition of DNP (8, 9). The fresh preparations also exhibit low rates of oxygen uptake even in the presence of substrate and increase their oxidative activity when ATP breakdown or turnover is increased (7-14). The mitochondria are considered as resting if they are operating at a rate of ATP turnover that is (a) near a low or minimal level and (b) capable of exhibiting a much higher turnover (cf. (9, 11)). The significance of these properties is suggested by our recent report on the intramitochondrial regulation of the oxidative rate (11).

The present study is a direct outgrowth of a report by Kielley and Kielley (7), referred to earlier, and is concerned with one of the properties that helps to characterize resting mitochondria in the above terms, namely, latent ATPase activity.

EXPERIMENTAL

Mitochondria of fed rats were prepared by the method of Schneider and Hogeboom (15). They were washed once with cold isotonic sucrose, and, after centrifugation, the loose fluffy layer above the mitochondria was

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1 The following abbreviations are used: ATP, adenosinetriphosphate; AMP, adenosine-5'-phosphate; ADP, adenosinediphosphate; IP, inorganic phosphate; DNP, 2,4-dinitrophenol; PCA, perchloric acid.

2 The term ATPase activity will be used to describe the release of inorganic phosphate from ATP regardless of the mechanism or number of enzymes involved.
swirled off. The mitochondria were then taken up for use in cold isotonic (0.25 M) sucrose. All preincubations were carried out in isotonic sucrose and all enzyme assays were done in reaction mixtures that were supplemented with enough sucrose to make the final mixture isotonic (9). This amount was calculated on the arbitrary basis that 0.25 M sucrose was taken as isotonic, and the other additions were assumed to furnish osmotic equivalents on the basis of 100 per cent ionization. Thus in Table I the last mixture contained 4 μM of ATP, 2 μM of CaCl₂, 4 μM of MgCl₂, and 470 μM of sucrose in a volume of 2.0 ml. All incubations were at 30°, with oxygen consumption measured in Warburg vessels and the assays for ATP breakdown performed in 13 x 100 mm. test-tubes.

In the experiments in which the individual nucleotides were to be measured, enzyme action was stopped by adding PCA at a final concentration of 0.5 M. After centrifuging the protein, the supernatant fluid was neutralized with KOH and stored in the refrigerator overnight. The insoluble potassium perchlorate was centrifuged and the supernatant fluid used for ion exchange chromatography. The enzymatic assays in which only inorganic phosphate was determined were stopped by adding PCA, as above, or trichloroacetic acid to a final concentration of 7 per cent.

The nucleotides in the medium were separated by the ion exchange method of Cohn and Carter (16) as modified slightly (17). Inorganic phosphate was measured by the following modification of the Lowry-Lopez method (18). To insure the correct final pH (18) the 1 per cent ammonium molybdate solution contained 0.05 M sodium acetate in order to buffer the ascorbic acid which was later added. Also, enough KOH was added to the acetate buffer solution to neutralize the amount of perchloric or trichloroacetic acid contained in the aliquot taken. In a comparison of this method with the Fiske and Subbarow procedure (10), experiments in which the “ATPase” activity of the mitochondria was determined gave identical results for inorganic phosphate output. It is of interest that low concentrations of cyanide increase the time needed for the development of the color in the Lowry-Lopez method, while similar amounts had no effect on color development in the Fiske and Subbarow procedure. It was found that either perchloric acid (final concentration 0.5 M) or trichloroacetic acid (final concentration 7 per cent) could be used to stop the enzymatic reaction without having any effect on the development of the color in the Lowry-Lopez method. However, when larger aliquots of the deproteinized filtrate were used (0.4 ml. in a final 3.0 ml. solution), both acids tended to give proportionally higher readings than those obtained with smaller aliquots (0.2 ml. or less in a final volume of 3.0 ml.).

The ATP and AMP were obtained either as the barium or sodium salts
from the Pabst Laboratories, and the barium salt of ADP was obtained from Dr. G. A. LePage.

**Results**

**Reaction Products Formed from Adenine Nucleotides**—When ATP or AMP is added to fresh resting mitochondria from liver of fed rats, the chemical changes that occur in the presence of magnesium ions and isotonic sucrose are small in magnitude. The mitochondria do not deaminate or dephosphorylate either compound and there is only a slight conversion of AMP to ATP and ADP. However, if both AMP and ATP are added to fresh resting mitochondria, there is a rapid formation of ADP, a corresponding decrease in both AMP and ATP, and a slight net conversion of AMP to ATP as shown by our earlier study on adenylate kinase (see especially Fig. 1, D (17)). Only the adenylate kinase activity was apparent, and, although adequate levels of all three nucleotides were present, the ATPase activity remained latent for the 90 minute incubation period and no net inorganic phosphate release occurred.

Although the activity of the latent ATPase can be released in a variety of ways, one of the simplest manipulations is to add DNP to the reaction mixture. In Fig. 1 it may be seen that under such conditions ATP is

![Diagram showing the effect of DNP on the dephosphorylation of ATP by rat liver mitochondria.](http://www.jbc.org/)

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**Fig. 1.** Effect of DNP on the dephosphorylation of ATP by rat liver mitochondria. Each tube contained the mitochondria from 25 mg. wet weight of liver, 9 μM of MgCl₂, 5.86 μM of ATP, 0.40 μM of ADP, 0.10 μM of AMP, and DNP at a final concentration of \(3 \times 10^{-6} \text{ M}\). The final volume of the reaction mixture was 3.0 ml. and the mixture was made isotonic by the addition of sucrose. The theoretical IP was obtained by calculating the increase on the basis of the changes in the nucleotide concentrations.
LATENT ADENOSINETRIPHOSPHATASE

rapidly broken down to ADP and AMP, while the total amount of adenine nucleotide remains constant. The breakdown of ATP results in inorganic phosphate formation, which is equal to the quantity that can be calculated from the changes in the amounts of the three nucleotides. This result shows that no net production of inorganic pyrophosphate occurs. Although it was clear from the earlier study (17) that the mitochondria contain enough adenylate kinase to account for the AMP production shown in Fig. 1, it cannot be decided from these data whether the inorganic phosphate arises partly from the conversion of ADP to AMP. This question appears to be answered in the negative, however, by the results obtained when ADP is added to fresh resting mitochondria in the presence of DNP and Mg$^{++}$. The system ([17] Fig. 1, C) is unable to dephosphorylate the ADP directly to AMP, and, even though the adenylate kinase rapidly converts ADP to a mixture of AMP and ATP, there is only a slow dephosphorylation of the latter. Thus the retardation of ATP disappearance and inorganic phosphate production in Fig. 1 is attributed in part to the presence of ADP, as indicated in a later section, and not to the lowering of the substrate concentration alone. These data establish that the sum of the three nucleotides remained constant and that the theoretical changes in inorganic phosphate agreed fairly well with the observed changes. The extent of side reactions was evidently small and the change in inorganic phosphate could be used as a measure of the extent of conversion of ATP to ADP, while the action of adenylate kinase should not affect the inorganic phosphate level except indirectly through its effect upon ADP concentration.

ATPase Activity in Fresh and Aged Mitochondria—The rate of ATP breakdown was measured in terms of inorganic phosphate released over various periods of time up to 4 hours at 30° in isotonic sucrose with various activators present. The activators were used in the combinations shown in Fig. 2 and each activator was used at the level that gave the maximal response. Fig. 2 includes data obtained with mitochondria stored at 30° for 120 minutes. The main purpose in presenting these data is to establish the validity of a 20 minute incubation period for the routine estimation of ATPase activity for either fresh or aged mitochondria. It may be seen that after about 40 minutes the initially rapid rates decline markedly, while at about this same time the latent ATPase of the fresh mitochondria in the presence of magnesium suddenly bursts into maximal activity and shows a rate comparable to that shown by the aged mitochondria or by the fresh mitochondria in the presence of both calcium and magnesium. The preparations of fresh mitochondria uniformly exhibit no ATPase activity whatever during a 20 minute incubation period, and, if the ATP substrate solution contained a small amount of ADP, there was a fraction
of a micromole less inorganic phosphate at 20 minutes than at time 0. The latter value amounted to about 0.4 \( \mu \text{M} \) per reaction tube and this was subtracted from all values obtained for the incubated tubes; hence, it was possible for the tubes containing Mg\(^{++}\) to exhibit a slight phosphate uptake at the 20 minute time point.

The endogenous oxygen uptake of these mitochondria is too small to measure accurately, and, even when 300 to 500 equivalent mg. of mitochondria were used, it was possible to observe only 10 to 20 \( \mu \text{l} \) of oxygen uptake in the first 10 minutes, followed by a decline in rate with or without DNP. The aged mitochondria can be centrifuged and resuspended without loss in ATPase activity, as will be shown later. The effect of the various activators can be seen more readily in Fig. 3.

**Effect of Preincubation in Sucrose**—Kielle and Kielley (7) had previously shown that aging for periods up to 45 minutes at 28° resulted in increases in the apparent ATPase activity in their standard test system which differed considerably from the one employed here and which contained Mg\(^{++}\) in all cases. Although they showed progressive increases in ATPase activity with increased preincubation time, their conditions apparently did not reveal a latent period in which there is no increase in ATPase activity and do not show the sudden way in which the latent ATPase activity becomes demonstrable as in our conditions. The present experiments were designed to show the changes induced in mitochondria by preincubation under the simplest conditions that could be arranged. This was done by preincubating in isotonic sucrose at 30° and then assaying for ATPase

![Figure 2: Effect of Ca\(^{++}\), Mg\(^{++}\), and DNP on release of IP from ATP in fresh and in aged mitochondria. Each tube contained the mitochondria (MW) from 25 mg. wet weight of liver and approximately 4 \( \mu \text{M} \) of ATP. When added, MgCl\(_2\) was at a final concentration of 0.002 M, CaCl\(_2\) at a final concentration of 0.001 M, and DNP at a final concentration of 3 \( \times 10^{-5} \) M. Sucrose was added as given in Fig. 1, except that the final volume was 2.0 ml.](http://www.jbc.org/content/232/3/897/figure/S1)
activity in the presence of the same combinations of activators shown in Fig. 2, with the time of incubation standardized at 20 minutes on the basis of the data shown in Fig. 2.

From the data in Fig. 3 it can be seen that the properties of the mitochondria in terms of their response to the activators of ATPase activity change progressively as the mitochondria are aged in sucrose at 30°. Three types of preparations are readily distinguishable: the "resting" mitochondria, which are obtained in the period from 0 to about 10 minutes, the "transition" mitochondria, which are obtained in the interval from 10 to 45 minutes, and the "aged" mitochondria, which are obtained in the period from 1 to 3 hours or longer.3

![Fig. 3. Effect of aging of mitochondria on release of IP from ATP. Each tube contained 25 mg. equivalent wet weight of liver mitochondria which had been incubated in isotonic sucrose at 30° for the times indicated. They were then centrifuged, taken up again in isotonic sucrose, and incubated in the assay medium for 20 minutes. The assay medium contained approximately 4 μM of ATP, and, when added, DNP at a final concentration of 3 × 10⁻⁵ M, CaCl₂ at a final concentration of 0.001 M, and MgCl₂ at a final concentration of 0.002 M. Sucrose and final volume as in Fig. 2.](http://www.jbc.org/)

3 It should be noted that many mitochondrial preparations previously reported in the literature as "aged" or without specification have, in fact, been in the period of transition, and it may be seen from Fig. 5 that the ratio of the response to Mg⁺⁺ to the response to DNP can change markedly during the time required to pipette samples when the mitochondria are in this condition. Moreover, the data obtained with various buffers suggest that in many buffers fresh mitochondria are not resting but pass into the stage of transition sooner than do the mitochondria in sucrose alone. For example, in the absence of Mg⁺⁺ and of inorganic phosphate, there is a breakdown of 0.43 μM of ATP, after 60 minutes at 30°, but, when inorganic phosphate buffer (pH 7.4) was added, 2.00 μM of ATP were broken down. On the other hand, the mitochondria can be maintained in the resting condition for much longer periods of time by including ATP in the reaction mixture as shown in Fig. 2 (7, 20), or by including oxidizable substrate in the reaction mixture under appropriate circumstances. Future work may be expected to increase the time during which the mitochondria can be maintained in the resting state.
The following points may be noted in the case of fresh mitochondria: (a) There appears to be a potential ATPase activity of about 4 μM of inorganic phosphate split off per 20 minutes, when the mitochondria from 25 mg. (wet weight) of liver are used. (b) This activity can be obtained with fresh mitochondria only by adding both calcium and magnesium ions. (c) Magnesium alone yields no apparent ATPase activity whatsoever in fresh mitochondria. (d) DNP or Ca++ alone produces sub-maximal stimulation. (e) Of considerable interest is the fact that the combination of DNP and Mg++ does not duplicate in magnitude the effect of Ca++ and Mg++. The decrease in responsiveness to DNP plus Mg++ prior to the stage of transition, as compared with the mitochondria at 0 minute preincubation, is real and in many experiments greater than that shown in Fig. 3.

The following points may be noted in the case of the aged mitochondria: (a) The potential ATPase activity is about the same as that in fresh mitochondria and may be demonstrated by adding Mg++ alone or with DNP. (b) DNP or Ca++ alone is quite ineffective in activating ATPase. (c) The combination of Ca++ and Mg++ is no more effective than Mg++ alone and may show slightly less activity.

The transition mitochondria show properties intermediate between those of the fresh and the aged mitochondria. It is important to note that the fresh mitochondria are active in oxidative phosphorylation, while the aged mitochondria are inactive in this respect (7, 20, 21). Moreover, the oxygen uptake of the fresh resting mitochondria is low and can be stimulated in the presence of oxidizable substrate by DNP and by Ca++ (11).

It seems likely that the lag shown in the Mg++ curve in Fig. 3 represents the time required to deplete the mitochondria of oxidizable substrate. The time required for the ATPase activity to be fully converted to the active form varies from about 30 to 60 minutes. The induction period has been studied carefully at 5 and 10 minute intervals during the first 40 minutes and has varied from 5 to 30 minutes in fed rats. However, when livers from two 24 hour fasted rats and one 24 hour regenerating liver were tested, the induction time was zero, with some ATPase activity evident even in the fresh mitochondria. The preincubation time required to reach the potential ATPase activity in the presence of Mg++ was shortened to 10 minutes in the case of the regenerating liver and one of the fasted livers. The ability to respond to DNP alone was lost within 10 minutes in all three preparations.

Effect of Preincubation with DNP—Studies were made on mitochondria that were given more complicated pretreatments than the incubation in

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4 But the breakdown of ATP to ADP and AMP by Ca++ plus Mg++ gives a similar pattern of nucleotide distribution as does the breakdown of ATP in the presence of DNP plus magnesium ion (Fig. 2).
sucrose alone as in Fig. 3. Mitochondria treated briefly (10 minutes at 0°) with DNP were recentrifuged to remove the bulk of the DNP, resuspended and allowed to stand for either 1 hour or 24 hours at 0° to permit further diffusion of DNP and mitochondrial contents such as Mg++ and unknown components (see "Discussion"), and then recentrifuged and tested in the various assay systems with the exception of DNP plus Mg++. The results (Table I) show that the properties of the mitochondria can be altered considerably without the loss of ATPase activity to the soluble form. The data show that the "latency" of ATPase may be lost simply by the loss of diffusible substances occurring with the operations of recentrifuging and resuspending; hence the addition of Mg++ alone elicits a response (2.28 μM of IP) nearly as great as that with Ca++ and Mg++ (3.04 μM of IP). Such mitochondria resemble those obtained from livers of fasting rats as described earlier. The variability in the response to Mg++ alone is probably a result of the transitional character of these preparations. The response to DNP alone clearly shows that the factors necessary for the response are lost more rapidly in the DNP-treated mitochondria than in the controls.

### Table I

**Effect of Adding DNP to Mitochondria Prior to Assay for Adenosinetriphosphatase Activity**

The data given are in micromoles of IP liberated from ATP in 20 minutes, although all assays included measurements after 20, 40, and 60 minutes to insure the validity of the data at 20 minutes. (Conditions as in Fig. 2.)

<table>
<thead>
<tr>
<th>Assay conditions (30°)</th>
<th>Control*</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNP</td>
<td>Aged control</td>
</tr>
<tr>
<td>Control, no additions</td>
<td>0.53</td>
<td>0.28</td>
</tr>
<tr>
<td>Ca++, 0.001 M</td>
<td>0.98</td>
<td>0.63</td>
</tr>
<tr>
<td>DNP, 3 × 10⁻⁵ M</td>
<td>1.65</td>
<td>0.53</td>
</tr>
<tr>
<td>Mg++, 0.002 M</td>
<td>2.28</td>
<td>1.44</td>
</tr>
<tr>
<td>Ca++ + Mg++ (see above)</td>
<td>3.04</td>
<td>2.86</td>
</tr>
</tbody>
</table>

* All the steps of the pretreatment carried out at 0°. Control mitochondria were prepared as 50 per cent suspension and diluted to 5 per cent suspension, allowed to stand for 10 minutes, recentrifuged, resuspended to 5 per cent suspension, allowed to stand for 1 hour to permit diffusion, recentrifuged, and resuspended to 12.5 per cent suspension; 0.2 ml. was assayed for ATPase activity. DNP mitochondria were given identical treatment except that they were exposed to 3 × 10⁻⁵ M DNP in sucrose during the 10 minute period. The aged control and aged DNP mitochondria were treated in a corresponding manner except that the 1 hour diffusion period was extended to 24 hours.
Effect of Variations in Concentration of Activators—In all cases the submaximal response shown by fresh mitochondria in the Mg++ and DNP mixture (as in Fig. 3) has consistently appeared. The explanation for this finding may be that DNP is unable to provide sufficient channels for ATP breakdown to counteract completely the effect of the oxidizing system, which resynthesizes ATP as long as any substrate is available. This explanation assumes that calcium in the presence of magnesium is able to provide channels for ATP breakdown that exceed those provided by DNP, and which thus completely negate any oxidative resynthesis of ATP. These data were obtained with $3 \times 10^{-5}$ M DNP and the fact that the response was submaximal (relative to Ca++ + Mg++) raised the question of whether higher levels of DNP would give a higher response. This was not the case. Under these conditions $3 \times 10^{-5}$ M DNP gave a response that was not exceeded by higher concentrations of DNP up to $50 \times 10^{-5}$ M which, in fact, gave slightly lower levels of activity. With concentrations of DNP at 0.5 or $1.0 \times 10^{-5}$ M the response was lower than with $3.0 \times 10^{-5}$ M DNP.

Thus the failure to elicit the potential ATPase activity cannot be attributed to a lack of DNP. In the same experiment in which DNP was varied, the potential ATPase activity was measured in the presence of Ca++ (0.001 M) and Mg++ (0.002 M) and was shown to be greater than that obtained with any level of DNP and Mg++, a duplication of the results shown in Fig. 3. Moreover, six levels of DNP in the range of 0.5 to $50 \times 10^{-5}$ M had no effect on mitochondria aged for 120 minutes and did not modify the effect of 0.002 M Mg++ on these mitochondria, again a verification of the results of Fig. 3 over a wide range of DNP concentrations.

The optimal concentration of magnesium was determined in mitochondria preincubated for 120 minutes, in which magnesium alone elicits the potential ATPase activity. The optimal concentration was $2 \times 10^{-3}$ M final with but a slight decrease shown in the range 0.5 to $5 \times 10^{-3}$ M and a slow decline between 5 and $15 \times 10^{-3}$ M. With the soluble ATPase of liver mitochondria, Kielley and Kielley (4) showed an optimal Mg++ concentration at from 3 to $8 \times 10^{-3}$ M. With fresh mitochondria in the presence of 0.001 M Ca++, the same result was given by 1 and $2 \times 10^{-5}$ M Mg++. In the absence of calcium, $2 \times 10^{-3}$ M Mg++ gave over twice as much activity as $5 \times 10^{-3}$ M Mg++ in the period between 40 and 60 minutes (see Fig. 2).

The optimal concentration of calcium was determined by use of fresh mitochondria in the presence of magnesium, since calcium has no effect on the aged mitochondria and since its effect in fresh mitochondria seems to depend on magnesium. The maximal effect was obtained with $0.5 \times 10^{-3}$ M Ca++ and no further increase or decline in activity was obtained in
the range of 0.05 to $2 \times 10^{-3}$ M; hence, the concentration chosen for most experiments was $1.0 \times 10^{-3}$ M. At $0.1 \times 10^{-3}$ M the activity was over 60 per cent of the maximal figure.

The addition of KCl or NaCl to the extent of 1.0 ml. of isotonic salt replacing an equal volume of isotonic sucrose had no effect on the control value for ATPase activity, but the effect of KCl on the other test systems was not studied. Recent work by Lardy and Wellman (14) shows that KCl strongly reinforces DNP in the absence of Mg$^{++}$ but has little effect on DNP in the presence of Mg$^{++}$. Studies comparing Ca$^{++}$ + Mg$^{++}$ with DNP + KCl have not been done.

Other investigators have used various buffers in the system but the present work was done with ATP and sucrose alone according to Lardy and Wellman (14). With 0.015 M histidine buffer at pH 7.4 the control curves showed a shorter lag than when histidine was absent. The presence of histidine accentuated the effect of calcium and DNP but the activating effect of magnesium was decreased. In order to test whether the results might be due to a lack of buffering capacity in the ATP, the system was tested by use of the calcium and magnesium combination with fresh mitochondria over a 4 hour period as in Fig. 2. The 4 $\mu$M of ATP were supplemented with 0, 4, and 8 $\mu$M of NaHCO$_3$. During the first 15 minutes the tubes containing bicarbonate were less active, but for the remainder of the incubation the rates were identical. With 4 $\mu$M of bicarbonate, the mixture remained at the original pH. Further studies with CO$_2$-bicarbonate systems (22) would be desirable as a means of buffering the mitochondria.

Effect of Calcium on Respiration—In explaining the data in Fig. 3 it was proposed that calcium can provide channels of ATP breakdown in excess of those provided by DNP. It has been shown by Hunter (8) that, in the oxidation of $\alpha$-ketoglutaric acid, one of the pathways of ATP resynthesis appears to be insensitive to DNP, and it was postulated that DNP provides channels of ATPase activity via "Y-phosphates" but not via an "X-phosphate" (8). If calcium can provide additional channels of ATPase activity, the X-phosphate system must be considered. An alternative explanation would be that calcium inhibits oxidation in this system. Fig. 4 shows that such is not the case. The addition of calcium ions to a system containing oxidizable substrate stimulates oxygen uptake in the range of concentrations up to the point of maximal stimulation of ATPase activity. The effect of DNP is similar and comparative studies of the effect on the ATP balance have been reported elsewhere (11). Calcium stimulates oxygen uptake by increasing ATP breakdown and does not increase ATP breakdown by inhibiting oxygen uptake.

Inhibition of ATPase Activity by ADP—It was reported by Kielley and Kielley (4) that the soluble ATPase of rat liver mitochondria is inhibited
by ADP. When ADP was added with ATP to fresh mitochondria in the presence of Mg++, and DNP, no inhibition of inorganic phosphate production could be obtained. However, this result was explainable in terms of the combination of adenylate kinase and ATPase activity. Since the former could be inhibited by fluoride (17, 23), it was possible to show that there is an inhibition of ATPase activity by ADP under the conditions in which adenylate kinase is inactive (Fig. 5). This result is pertinent to the explanation for the data shown in Fig. 1. The data obtained with fresh mitochondria thus support the finding with a soluble extract (4).

![Graph](image)

**Fig. 4.** Effect of Ca++ on oxidative rate of rat liver mitochondria. Each Warburg flask contained the mitochondria from 160 mg. wet weight of liver, 30 µM of phosphate buffer, pH 7.4, 10 µM of fumarate, 20 µM of pyruvate, 9 µM of MgCl₂, approximately 3 µM of ATP, cytochrome c at a final concentration of 6.7 × 10⁻⁶ M, and CaCl₂ as indicated. Sucrose and final volume as given in Fig. 1.

**Effect of DNP on Respiration in Absence of Mg⁺⁺—**In fresh resting mitochondria with oxidizable substrate in the presence of ATP and Mg⁺⁺, the ATPase activity is latent and the oxygen uptake is low. The respiration is able to maintain the ATP level under these conditions (Fig. 6). When Mg⁺⁺ is omitted from the reaction mixture, the respiration is no longer coupled to the maintenance of ATP and there is sufficient increase in ATP breakdown to stimulate respiration. However, this respiration is not maintained because the relative ATP breakdown is excessive. When DNP is added to the system in the absence of magnesium, there is a maximal stimulation of respiration, but, again, the respiration rapidly declines as the ATP is depleted. It might be inferred that in addition to causing the breakdown of high energy phosphate DNP increases the availability of
Mg++. However, with no Mg++ in the medium the mitochondria rapidly lose their endogenous supply, and respiration is uncoupled from phosphorylation and declines. Finally, the effect of DNP in the presence of added Mg++ is shown (Fig. 6). The DNP provides a rapid breakdown of ATP as shown by the stimulation of oxygen uptake (cf. (11)), but the magnesium

permits the coupling of part of the energy of oxidation and the ATP is not depleted at the rate that it would be in the absence of oxidation and, in fact, is depleted much more slowly.

**ATPase Activity in Whole Homogenates of Various Tissues**—Studies on whole homogenates of liver can be summarized briefly by saying that the responses observed with mitochondria, as described by Figs. 2 and 3, also are shown by whole homogenates. This is because the result in the latter is dominated by the mitochondria therein. Although the nuclei and the supernatant material from the mitochondria (fluffy layer, microsomes, and
soluble fraction) are perceptibly affected by the activators, the response is essentially independent of preincubation, and the magnitude of the activity is small in comparison with that of the mitochondria. One difference is that in the mitochondria the ATPase activity in the control or in the Mg²⁺-activated systems is essentially zero, while in the whole homogenate the activity of the remaining fractions, which is not zero under these conditions, can be seen. This point is illustrated in Table II which includes assays of several other tissues. The data for liver show that the Mg²⁺-

### Table II

**ATPase Activity of Whole Homogenates**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Amount mg. wet weight</th>
<th>μM IP per 20 min.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>15</td>
<td>0.29 0.80 2.3 3.4 1.8</td>
</tr>
<tr>
<td>Kidney</td>
<td>15</td>
<td>0.85 4.5 6.0 3.1</td>
</tr>
<tr>
<td>Hepatoma 98/15</td>
<td>20</td>
<td>0.62 2.5 2.1 3.1 1.3</td>
</tr>
<tr>
<td>Hepatoma 98/15†</td>
<td>20</td>
<td>0.70 2.8 2.8 4.2 3.9 1.3</td>
</tr>
<tr>
<td>Flexner-Jobling carcinoma</td>
<td>20</td>
<td>0.50 2.2 1.4 2.3 2.4 0.8</td>
</tr>
<tr>
<td>Flexner-Jobling carcinoma †</td>
<td>100</td>
<td>0.07 0.40 0.40 0.5 0.41 0.07</td>
</tr>
<tr>
<td>Flexner-Jobling carcinoma †</td>
<td>250</td>
<td>0.41 1.0 0.61 1.5† 0.93 0.55</td>
</tr>
<tr>
<td>Human fibrosarcoma</td>
<td>50</td>
<td>1.75 2.50 2.0 2.75 3.5 2.9</td>
</tr>
</tbody>
</table>

* Observed values given. All assays included measurements at 10, 30, and 60 minutes in order to be sure that the 20 minute value was on a smooth curve. The values obtained by doubling the 10 minute values would be higher in all cases.

† Mitochondria preparation, from given wet weight of tissue.

‡ 1000 mg. of the same preparation gave only 1.8 μM here.

activated system remains far below the activity when both Mg²⁺ and Ca²⁺ are present and below the values for Ca²⁺ or DNP alone. These effects are due to the responses of the mitochondria, and the activity of the Mg²⁺ system is low because the mitochondrial ATPase is latent. Such is not the case with the other tissues studied, in which Mg²⁺ causes a large activation that approaches the effect of Ca²⁺ plus Mg²⁺ and exceeds the effect of DNP alone.

It is of interest that in the case of the homogenate of the Flexner-Jobling carcinoma in which the capacity for oxidative phosphorylation is rapidly lost in the absence of fluoride (24) the response to DNP was very slight, and the same was true for the tumor mitochondria. In the latter there was a lack of proportionality with respect to tissue concentration. In both
the Flexner-Jobling carcinoma and the 98/15 mouse hepatoma, addition of 0.01 M fluoride to the Mg++ system reduced the activity to less than that of the control (not shown in Table II).

DISCUSSION

The data presented in this report suggest that the phenomenon of latent ATPase may be limited to the mitochondria of liver as presently prepared and may be restricted to mitochondria that are obtained from fed animals under conditions that minimize the time during which the mitochondria are allowed to stand in sucrose.

Rather than to search for other possible sources of mitochondria that would exhibit similar properties the present work has been largely confined to a study of the properties of mitochondria that contain latent ATPase and which we have termed resting, since these mitochondria may be of great importance in developing our knowledge of intracellular organization. Elsewhere it has been shown that such mitochondria can oxidize substrates of the Krebs cycle at a rate that depends on that of ATP breakdown, with the maximal rate of oxygen uptake occurring at a rate of ATP breakdown that provides an adequate supply of ADP and AMP without depleting the ATP of the medium. In the oxidative study (11), the oxidative rate was altered by changing the rate of ATP breakdown under circumstances in which ATP resynthesis was occurring as a result of oxidative processes. In the present work two of the factors that promote ATP breakdown have been studied in the absence of added oxidizable substrates so that their effect as activators of ATP breakdown would be uncomplicated by oxidative resynthesis. The data suggest, however, that the ATP-generating phenomena are not eliminated from the resting mitochondria and that they may be an important component of the ATP-hydrolyzing mechanism.

The transition of fresh resting mitochondria into aged mitochondria with a gain in responsiveness to Mg++ and a concomitant loss in responsiveness to DNP and Ca++ implies the loss of certain components from the mitochondria during this process and recent experiments have shown that the transition is correlated with the loss of substances that absorb light maximally at 260 mμ. The further interpretation of the present data may depend upon the nature of the substances liberated from the mitochondria and the mechanisms involved in their release.

The present findings show that the response of mitochondrial ATPase to calcium and magnesium ions is strongly dependent upon the condition of the mitochondria and provide a possibly adequate explanation for some of the conflicting reports in the literature (3). The complicated nature of ATPase activity is now more readily appreciated than it was 10 years ago.

A preliminary report has appeared (25) and further studies are in progress.
earlier (2), but our knowledge of the fundamental nature of ATPase activity is still not much more advanced than it was when the assay technique of DuBois and Potter was first reported (2), and the comments made at the time on the nature of ATPase activity are still applicable. Although the present data suggest that the use of calcium alone as an activator of ATPase activity is clearly undesirable, it is at present not possible to propose a standard assay method for one or more of the components of the so called ATPase activity. It seems likely that the ATPase activity of the various cell fractions may have different optimal conditions as well as functions, and the techniques of cell fractionation appear to be a highly desirable preliminary step in the study of the nature of this activity.

SUMMARY

1. Freshly prepared rat liver mitochondria in isotonic sucrose were shown to possess very little ATPase activity at 30° either in the presence or absence of magnesium ions when observed for short periods of time. They did not deaminate or dephosphorylate AMP, ADP, or ATP, but were active in bringing about an equilibrium between a mixture of these compounds. Such mitochondria may be referred to as resting when they can be shown to be operating at a low level of ATP turnover and to be capable of a much higher rate of turnover.

2. When calcium ions or DNP is added to a suspension of resting rat liver mitochondria, there is a marked increase in ATPase activity, but the maximal response is obtained only when magnesium ions are also present.

3. When fresh rat liver mitochondria are maintained at 30° in the presence of sucrose alone, they lose the properties of resting mitochondria and are no longer capable of responding to DNP or calcium ions. The aged mitochondria now exhibit maximal ATPase activity in the presence of magnesium ions alone.

4. When fresh rat liver mitochondria are preincubated at 30° in the presence of DNP and then washed free of the DNP, the maximal ATPase activity can be obtained only in the presence of added magnesium ions.

5. The optimal concentrations for the activations noted were 3 \times 10^{-5} \text{ M} DNP, 2 \times 10^{-3} \text{ M} magnesium ion, and 0.5 \times 10^{-2} \text{ M} calcium ion.

The important contributions by Hunter (8) and by Lardy and Wellman (12, 14) have provided attractive possibilities for the explanation of the DNP phenomenon, and the most recent paper by Lardy and Wellman (14) seems compatible with the present data. Their proposals seem to imply that an enzyme such as the ATPase recently isolated (4) does not react with water in the resting mitochondria, and we regard the enzyme in the aged mitochondria as a similarly altered system (cf. (2)). However, the proof for these concepts remains for the future and further discussion here seems unwarranted.
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