The Control of Uncoupler-activated ATPase Activity in Rat Liver Mitochondria by Adenine Nucleotide Transport

THE EFFECT OF GLUCAGON TREATMENT*

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Acute treatment of rats with glucagon increased the $V_{max}$ but did not change the $K_m$ (ATP) of uncoupler-activated ATPase in subsequently isolated hepatic mitochondria. The hormonal stimulation was evident in mitoplasts but not in submitochondrial particles nor after lysis of the mitochondria. The rate of $Pr_ATP$ exchange of intact mitochondria was also increased by glucagon treatment. The hormonal stimulation of ATPase was dependent on concentration of the uncouplers, being absent at minimally effective concentrations while high concentrations inhibited the ATPase. Inhibitors of adenine nucleotide transport decreased ATPase activity without evidence of sigmoidicity in the response curves and produced linear Dixon plots indicating that the ATPase was limited by the rate of adenine nucleotide transport. Glucagon treatment did not change the number of binding sites for transport inhibitors. Glucagon stimulated the rate of transport of ATP activity when measured in the presence of exchangeable adenine nucleotides within mitochondrial nucleotides appears to explain a number of the effects of hormones on mitochondrial functions including the stimulation of uncoupler-activated ATPase activity.

Yamazaki et al. (1) have reported that brief treatment of intact rats with glucagon altered the liver so that subsequently isolated mitochondria displayed an enhanced rate of ATPase activity when measured in the presence of a protonophoric uncoupler. We have recently studied this phenomenon using isolated hepatocytes, thus eliminating extrahepatic factors (2). We have found that the activation of the ATPase is as rapid as the stimulation of gluconeogenesis, occurring within 2 min after hormone addition, and is sensitive to the same physiological doses of hormones as are the stimulations of pyruvate carboxylation and gluconeogenesis (3). No data are available as yet as to the molecular mechanism of the hormonal stimulation of the ATPase or as to which kinetic parameters of the enzyme are altered by glucagon pretreatment. Of the mitochondrial functions stimulated acutely by hormones, the effect on the ATPase appeared to be potentially more amenable to an in-depth study than most others inasmuch as understanding of the ATPase reaction and the ATPase enzyme complex is advancing rapidly. We, therefore, decided to study the acute response of the hepatic mitochondrial ATPase to glucagon by delineating those characteristics of the reaction that were considered likely to be of aid in elucidating the mechanism of hormonal stimulation.

MATERIALS AND METHODS

FCCP was obtained from Pierce Chemical Co. ATP, DNP, antimycin, oligomycin, potassium stearateslydite, digitonin, glucagon, bovine serum albumin, and pyruvate kinase were purchased from Sigma. Carboxylmethyltriaclysteine, triethanolamine, and lactate dehydrogenase were obtained from Boehringer Mannheim and Triton X-100 from Beckman Instruments. [8-14C]ATP was purchased from Amersham-Searle. BKA and TTFB were generous gifts of Professor W. Berends, Delft University of Technology, and Professor Henry Lardy of the University of Wisconsin, respectively.

Fed, male Wistar strain rats weighing between 200 and 350 g were used. The animals were anesthetized by an intraperitoneal injection of pentobarbital (60 mg/kg) and were immediately treated subcutaneously with 100 µg of glucagon or its injection vehicle, saline-0.05% bovine serum albumin. After 20 min, the livers were removed rapidly and mitochondria prepared by differential centrifugation in 0.3 M sucrose, 5 mM Tes, 1 mM EDTA, pH 7.4 (STE), as previously described (4), except that the mitochondrial fraction was washed once in these experiments.

ATPase activity was determined in most experiments by the measurement of inorganic phosphate released using the Fiske-SubbaRow method (5). The medium was essentially that of Weiner and Lardy (6): 250 mM sucrose, 15 mM KCl, 10 mM triethanolamine, pH 7.4, 0.1 mM EDTA, and 0.5 mM Tes. Unless indicated otherwise, the temperature was 30°C, protein 0.5 mg/ml, DNP 0.2 mM, and ATP 4 mM. The reaction was initiated by the addition of mitochondria or ATP and allowed to proceed for 8 min before termination with trichloroacetic acid added to a final concentration of 10%.

For experiments designed to measure kinetic functions, mitochondria (0.25 mg/ml) were incubated for 2 min at 30°C with varying concentrations of ATP in the presence of 2 mM MgCl₂. The reaction was initiated by the addition of 0.2 mM DNP and was terminated after 30 s with perchloric acid. The reaction was found to proceed linearly over this time interval. Inorganic phosphate was measured by the technique of Itaya and Ui (7).

Measurements of kinetic functions of disrupted mitochondria were made after mitochondria were lysed by the addition of 0.5 mg of Triton X-100/mg of mitochondria. In these experiments, ATPase was

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1 The abbreviations used are: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; DNP, 2,4-dinitrophenol; BKA, bongkrekic acid; TTFB, tetrachlorofluoromethylbenzimidazole; EGTA, ethyleneglycol bis[β-aminoethylether] N,N,N',N'-tetraacetic acid; STE, 0.3 M sucrose, 5 mM Tes, and 1 mM EDTA, pH 7.4; Mops, 4-morpholinopropanesulfonic acid; Tes, N-[Tris(hydroxymethyl)methyl-2-amino]ethanesulfonic acid.
measured spectrophotometrically by coupling to NADH oxidation via pyruvate kinase and lactate dehydrogenase (8). Sufficient Mg\(^{2+}\) was present in this assay to bind all ATP; the concentration of added ATP was considered to be the substrate concentration. Kinetic functions were estimated by the Lineweaver-Burk technique.

ATP \(_{i}\) exchange was measured at 30°C by the procedure of Pullman (9). The incubation medium was that used in the ATPase assay.

The rate of transport of ATP was measured by following the accumulation of \([8-\text{\^{14}}C]\)ATP in mitochondria at 10°C. The incubation medium that was used for ATPase measurements plus 80 \(\mu\text{M}\) \(\text{[8-\text{\^{14}}C]}\text{ATP}\) (approximately 5000 cpm/nmol), 13 \(\mu\text{g}\) of oligomycin, and 0.2 \(\text{mM}\) DNP, protein was 0.5 mg/ml. Transport was arrested with 3 volumes of ice-cold STE containing 4 \(\text{mM}\) MgCl\(_2\) and 0.2 mm potassium atractyloside. The mitochondria were trapped on a 0.4-pore Millipore filter and washed three times with the "stop" solution.

The filter was air dried and then added to a toluene-0.4\% 2,5-diphenyloxazole mixture for counting.

Mitoplasts (mitochondria that have had the outer membrane removed) were prepared by a digitonin technique (10). Satisfactory removal of the outer membrane was indicated by the loss of at least 94\% of the monamine oxidase activity (11). Submitochondrial particles were prepared by sonic disruption of mitoplasts suspended in water followed by removal of residual mitoplasts by centrifugation at 17,000 \(\times g\). The submitochondrial particles were collected at 100,000 \(\times g\) and washed once before using.

ATP and ADP were measured fluorometrically in neutralized perchloric acid extracts (12). Protein was determined by the technique of Lowry et al. (13). Respiration was measured in 125 mM KCl, 50 mM Mops, 1 mM EGTA, 5 mM K\(_2\)HPO\(_4\), and 0.2 mM MgCl\(_2\), pH 7.0. The MgCl\(_2\) was omitted in experiments measuring respiration of mitoplasts.

### RESULTS

**Effect of Glucagon Treatment on Basal and DNP-stimulated ATPase**—Treatment with glucagon produced a stimulation of uncoupler-activated ATPase in mitochondria when assayed either in the absence of presence of 1 mM Mg\(^{2+}\) (Table I). As can be seen in this table, there was no evidence of stimulation of the basal ATPase in the absence of Mg\(^{2+}\), and in the presence of Mg\(^{2+}\), there was, in fact, significantly less ATPase activity in the mitochondria from glucagon-treated rats. When the mitochondria were disrupted by detergent, the ATPase activity was negligible in the absence of added Mg\(^{2+}\) (data not shown). In the presence of Mg\(^{2+}\), the effect of the hormone was absent, and the ATPase activity of both the control and glucagon-treated tissue approached the rate of uncoupler-dependent ATPase of intact mitochondria from hormone-stimulated animals. Thus, there was clearly a restraint imposed on the enzyme in intact mitochondria that was relieved by either hormone treatment or by the removal of membrane barriers.

Because the stimulatory effect of glucagon on ATPase was lost when the mitochondria were lysed with detergent, an experiment was performed to ascertain what level of structural organization was required to demonstrate the hormone effect. Mitoplasts and submitochondrial particles were prepared from control and glucagon-stimulated mitochondria as described under "Materials and Methods," and basal and uncoupler-activated ATPase were assayed at each of these stages of mitochondrial disorganization. The results are presented in Table II together with respiratory data from the same preparations. We have previously shown that submitochondrial particles prepared from glucagon-stimulated mitochondria retain an enhanced rate of respiration (14). The respiratory data, therefore, served as a positive control relative to the ATPase to demonstrate the presence of a hormone effect in the various fractions. It is evident from this table that removal of the outer membrane of the mitochondria did not significantly alter the effect of glucagon on ATPase activity. However, this effect was lost at the level of the submitochondrial particles when the inner mitochondrial membrane was disrupted and inverted. In contrast, as previously reported, a hormonal stimulation of respiration persisted in these preparations.

**Kinetic Functions of the ATPase**—The experiments of Table I were carried out using 4 mM ATP, a concentration of ATP presumed to be nearly saturating for the enzyme complex. The finding of a stimulation with this concentration of ATP suggested that a change in maximal velocity of the reaction is produced by hormone treatment. To determine whether this is indeed the case and to see whether hormonal stimulation also alters the affinity of the enzyme complex for ATP, kinetic studies were carried out. In this group of experiments, the technique of Verdouw and Bertina (15) was used in which Mg\(^{2+}\) is complexed with the added ATP, and the free ATP, considered to be the true substrate, is calculated from the Mg\(^{2+}\)-ATP association constant reported as 10\(^{-4}\)/M (16).

Data from six experiments were combined and are presented in Fig. 1. From these experiments, the \(V_{\text{max}}\) for control mitochondria was determined to be 243 ± 13 nmol/min/mg and that of the mitochondria from hormone-treated rats 441 ± 33 nmol/min/mg. The \(K_m\) for controls was 18 ± 4 \(\mu\text{M}\) and that for the hormone-stimulated mitochondria 18 ± 2 \(\mu\text{M}\). The data, therefore, indicated that there was an increase in the \(V_{\text{max}}\) of the enzyme complex as anticipated but no significant change in the affinity of the enzyme for ATP. These data are consistent with those of Verdouw and Bertina (15), who reported values ranging between 5 and 29 \(\mu\text{M}\) for the ATP \(K_m\). They also noted the presence of a higher \(K_m\) ranging between 58 and 119 \(\mu\text{M}\).

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
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<tbody>
<tr>
<td>The effect of Mg(^{2+}) and lysis on basal and uncoupler-activated mitochondrial ATPase</td>
</tr>
</tbody>
</table>

ATPase was measured as described under "Materials and Methods." Where indicated, 0.025% Triton X-100 was present. Results are given as means ± standard error for five experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATPase activity</th>
<th>(\text{nmol/min/mg})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Basil</td>
<td>+DNP</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Basil</td>
<td>+DNP</td>
</tr>
<tr>
<td>20 ± 2</td>
<td>263 ± 19</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>18 ± 2</td>
<td>395 ± 23</td>
<td>21 ± 6</td>
</tr>
</tbody>
</table>

\(a\) Group comparisons, two-tailed t test, \(p < 0.005\).

\(b\) Group comparisons, two-tailed t test, \(p < 0.025\).

\(c\) Group comparisons, two-tailed t test, \(p < 0.001\).

**P-ATP Exchange Reaction**—The P\(_i\)-ATP exchange reac-
Control of ATPase by ATP Transport and Glucagon

Table II

Hormonal stimulation of ATPase exhibited by mitochondria and mitoplasts but not by submitochondrial particles

The data represent the means ± standard errors of four or five experiments. Respiration of mitochondria and mitoplasts was measured with 5 mM glutamate and 0.5 mM malate as substrates. Respiration of submitochondrial particles was measured using 1 mM NADH as substrate in the presence of 5 μg of oligomycin/ml. DNP (0.2 mM) was the uncoupler for ATPase; FCCP (0.25 μM) was the uncoupler for respiration.

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Treatment</th>
<th>ATPase</th>
<th>Respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Uncoupled</td>
<td>Basal</td>
</tr>
<tr>
<td></td>
<td>nmol/min/mg</td>
<td></td>
<td>nmol O/min/mg</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Control</td>
<td>26 ± 2</td>
<td>213 ± 23</td>
</tr>
<tr>
<td></td>
<td>Glucagon</td>
<td>26 ± 2</td>
<td>383 ± 24*</td>
</tr>
<tr>
<td>Mitoplasts</td>
<td>Control</td>
<td>35 ± 3</td>
<td>167 ± 12</td>
</tr>
<tr>
<td></td>
<td>Glucagon</td>
<td>34 ± 3</td>
<td>398 ± 16*</td>
</tr>
<tr>
<td>Submitochondrial</td>
<td>particles</td>
<td>Control°</td>
<td>1523 ± 157</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucagon</td>
<td>1458 ± 277</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>795 ± 32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucagon</td>
<td>809 ± 84</td>
</tr>
</tbody>
</table>

a Pair comparison, one-tailed t test, p < 0.001.
b Pair comparison, one-tailed t test, p < 0.025.
° ATPase activity was measured in particles at two concentrations of Mg2+, the first two rows at 2 mM, the second two rows at 4 mM.

Fig. 1. Substrate velocity relationships (A) and Lineweaver-Burk plots for uncoupler-activated mitochondrial ATPase (B). ATPase was measured over a 30-s interval at 30°C as described under "Materials and Methods." The data represent the means of six experiments ± standard errors in the substrate-velocity portion of the figure. Velocity is expressed as nanomoles of ATP hydrolyzed/min/mg. ATP was added in concentrations ranging from 0.37 to 1.2 mM. In this figure, substrate concentrations are given as free ATP calculated as indicated in the text. In the Lineweaver-Burk portion of the figure, the lines were drawn by a linear regression analysis. □, control; ■, glucagon treated. Vmax, control = 243 ± 13 nmol/min/mg; Km = 18 ± 4 μM; Vmax, glucagon = 441 ± 33 nmol/min/mg; Km = 18 ± 2 μM.

Fig. 2. The effect of different concentrations of uncouplers on the ATPase response to glucagon. ATPase was measured as described under "Materials and Methods." Points represent the means ± standard errors of four experiments except where the numbers in parentheses indicate otherwise. Points presented without standard error bars had standard errors smaller than the symbols used. ○, control; ■, glucagon treated.

Sensitivity to Uncouplers—The responses of mitochondria to different concentrations of three uncouplers are presented in Fig. 2. As can be seen, minimally effective concentrations of uncouplers did not permit expression of the hormonal stimulation, presumably because the rate-limiting step at these concentrations of the uncouplers was the transfer of protons into the mitochondrial matrix. As the concentration of the uncouplers was increased, ATPase activity in glucagon-stimulated mitochondria rose faster than that of control mitochondria. Then, as the level of uncoupler was raised further, there was a striking decline in ATPase activity in the control mitochondria, while the activity in glucagon-stimulated mitochondria was more resistant or (as with FCCP) actually increased. At these concentrations of uncoupler (0.3 μM for FCCP, 0.2 mM for DNP, and 1 μM for TTFB), the most dramatic differences between the activity of the two groups of mitochondria were seen. Finally, still higher concentrations of the uncouplers led to severe inhibition of ATPase in the glucagon-stimulated as well as the control mitochondria. Inhibition of ATPase by high concentrations of uncouplers has been recognized for some years (18), but studies on the mechanism of this phenomenon are contradictory (15, 19). Transport of ATP into the mitochondria should be facilitated by the progressive decrease of the proton gradient (20) that presumably occurs as uncoupler concentrations are increased, but it may be inhibited by very severe de-energization of the mitochondria. It is apparent also from these figures that the maximal stimulation by FCCP is lower than that of DNP. These titrations were made with different groups of mitochondria for each uncoupler; nevertheless, we have consistently seen a much weaker response to maximally effective concentrations of FCCP than to DNP.

Effects of Antimycin and Oligomycin—Yamazaki et al. (1) noted that antimycin, in concentrations many times greater than required to block electron transport, inhibited uncoupler-dependent ATPase to a greater extent in control mitochondria than in glucagon-stimulated organelles. We re-examined this phenomenon using a wider range of concentrations of antimycin. It is evident from the data of Fig. 3 that hormone treatment produces a resistance to the inhibitor effects of the antibiotic. It can be seen, however, that as the dose of the...
inhibitor is increased from 3 to 30 μg/mg of protein, the activity of the ATPase in the stimulated mitochondria falls rapidly and approaches the control rate. We are unaware of studies explaining the mechanism by which antimycin inhibits ATPase at high concentrations although the phenomenon has been reported before (6). The different sensitivity of the treated and control groups of mitochondria is related to the action of antimycin and does not appear to be a general response of the mitochondria themselves to inhibitors. This is evident from the second portion of Fig. 3 which presents data from experiments in which oligomycin was used as the inhibitor. It can be seen that low doses of oligomycin affected both groups in a parallel manner with no evidence of a decreased sensitivity in the hormone-stimulated mitochondria.

**Effects of Inhibitors of Adenine Nucleotide Translocase—**

There has been a long standing controversy as to whether adenine nucleotide transport is rate limiting for uncoupler-dependent ATPase. A number of workers have favored the concept that transport is rate limiting (15, 21, 22), while Mitchell and Moyle (23), on the basis of the sigmoidicity of an atractyloside titration, reported that transport was not rate limiting for ATPase under the conditions of their experiment. In order to explore the possibility that hormonal stimulation of ATPase takes place at the transport step, we performed several experiments with inhibitors of transport.

Uncoupler-activated ATPase was assayed in mitochondria with increasing doses of inhibitors present. The results of experiments with atractyloside and carboxyatractyloside are presented in Fig. 4. It can be seen in these experiments that there was no suggestion of sigmoidicity in the response to these inhibitors in contrast to the experiment reported by Mitchell and Moyle (23). In Fig. 5, it can be seen that there was also no evidence of sigmoidicity with bongkrekic acid as inhibitor. A more stringent test for association of the activity of the ATPase and the inhibition of transport produced by these agents is the use of the Dixon plot. In the insets of Fig. 4, it can be seen that such plots of these data, taken over the range of zero to approximately 60% inhibition, are linear, indicating that ATPase is fully controlled by these inhibitors of transport throughout this range. Were some other factor, for example, the reactivity of the enzyme itself, to become limiting as the inhibition of transport approached zero, one would expect the reciprocal of the reaction velocity to fail to decrease linearly as the inhibitor is decreased and to drift higher toward a velocity at zero inhibitor concentration that is determined by the limiting component.

Titrations of inhibitors also provide data for an additional analysis. The concentration of binding sites for the inhibitors, which presumably relate directly to the number of accessible transporter molecules, can be estimated by use of ligand-conservation plots (24, 25). In these plots, presented as insets of Fig. 5, the value of y at \((1 - i)^{-1} = 1\) equals the concentration of binding sites. In five experiments, carboxyatractyloside binding sites were estimated to be \(5.8 \pm 0.9 \times 10^{-7}\) mol/g in control mitochondria and \(5.4 \pm 0.8 \times 10^{-7}\) mol/g in glucagon-stimulated organelles. The corresponding binding sites for bongkrekic acid were \(5.1 \pm 1.0 \times 10^{-7}\) mol/g and \(5.1 \pm 1.1 \times 10^{-7}\) mol/g. Thus, there was no significant difference in the concentration of inhibitor binding sites between control and glucagon-stimulated mitochondria, nor was there a significant difference between inhibitors. Our data are close to the recent estimate of rat liver binding sites using oxidative phosphorylation as the function inhibited by atractyloside, \(3.8 \times 10^{-7}\) mol/g (25), and to the 1 to \(3.5 \times 10^{-7}\) mol/g estimates of
binding sites for atractyloside and carboxyatractyloside found using inhibition of transport (20). If the rate of transport is not in excess of that of the ATPase, then the apparent number of binding sites as measured by a decrease in ATPase activity to that determined by measuring ATP transport. Hormone treatment did not change the available binding sites for inhibitors of adenine nucleotide transport and, thus, presumably did not make additional carrier molecules available.

Transport Studies—A direct measure of transport was performed using uptake of $[^{14}C]$ATP at 10°C in the presence of uncoupler. Other aliquots of the mitochondria were used to measure ATPase activity at the same temperature. For 10 experiments, the mean values of ATPase activity were control $26 \pm 1$ nmol of ATP hydrolyzed/min/mg; and glucagon $41 \pm 1$ nmol of ATP hydrolyzed/min/mg; $p < 0.001$. Fig. 6 presents the data from the transport experiments. In Fig. 6A, the accumulation of $^{14}C$ in the mitochondria during the 15-s experimental period is shown. It can be seen that the initial rate of accumulation is greater in glucagon-stimulated mitochondria than in controls. This effect of glucagon treatment on transport has been observed previously by Bryla et al. (26). Equilibration values of $^{14}C$ accumulation were determined by allowing uptake to continue for 2 min, and rate constants were calculated from the slope of the curve when ln (maximal accumulation/maximal accumulation = accumulation at time $t$) was plotted against time. In Fig. 6B, this plot is given as one line for both control and glucagon-stimulated mitochondria, as the mean values and standard errors of the two groups were not significantly different; the rate constants were 4.6 $\pm$ 0.3 min$^{-1}$ for both groups. The initial rates of transport were $38 \pm 2$ nmol/min/mg for control mitochondria and $62 \pm 3$ nmol/min/mg for glucagon-stimulated mitochondria. As the rate constants were identical, the calculated difference in initial rates was the consequence of a difference in equilibrium values of labeling.

The initial rates of ATP transport of both control and stimulated mitochondria are approximately 50% higher than the rates of ATPase. The reasons for this apparent discrepancy are not clear to us, but it should be pointed out that the ATPase reaction is measured over an 8-min period in contrast to the 5- to 15-s time intervals used in transport studies. This or some other factor may cause the disparity in estimated rates.

The difference in equilibrium labeling between control and glucagon-stimulated mitochondria suggested that there was a difference in concentration of exchangeable nucleotides present in the two groups of mitochondria. A series of mitochondrial preparations were assayed for ATP and ADP to investigate this possibility, and the results are given in Table III. It can be seen that the mitochondria from glucagon-treated rats had significantly more exchangeable adenine nucleotides, i.e. ATP and ADP than controls; the difference in ATP concentration was particularly striking. The 52% greater concentration of exchangeable nucleotides in the glucagon-stimulated mitochondria can account for the greater rate of $[^{14}C]$ATP transport by permitting a true increase in transport rate via a mass action effect and also by providing a larger pool for the entering labeled ATP, thereby making the label statistically less subject to outward transport. The increase of exchangeable nucleotides present in glucagon-treated mitochondria is comparable to the 64% increase in equilibrium labeling of stimulated mitochondria with $[^{14}C]$ATP and the 58% increase in ATPase found when assayed at the same temperature.

In recent experiments not presented here, we have found an increase in the total adenine nucleotide content of mitochondria prepared in 0.3 M sucrose from glucagon-treated rats. This indicates the phenomenon is not peculiar to mitochondria prepared in the STE medium used in this study.

### DISCUSSION

The data of this paper support the concept that the uncoupler-activated ATPase of rat liver mitochondria is limited by the rate of transport of ATP into the mitochondria. The demonstration by our data of a lack of sigmoidicity in the response of ATPase activity to transport inhibitors atractyloside, carboxyatractyloside, and bongkrekic acid means that inhibition of adenine nucleotide transport, no matter how small the inhibition, is always accompanied by a corresponding decrease in ATPase activity. The Dixon plots provide objective presentations of this fact. The observation that the ATPase activity of submitochondrial particles and lysed mitochondria is greater than that of uncoupled intact mitochondria is explicable by the fact that in both of these preparations there is no membrane barrier between exogenous ATP and the enzyme complex through which the ATP must be transported. Additional confirmation of the concept of limitation by transport was the finding that ATPase activity was proportional to the concentration of exchangeable adenine nucleotides within the mitochondria when control and glucagon-stimulated mitochondria were compared. Although the rate constants for transport were identical for the two groups, the difference in the size of the exchangeable pool of nucleotides

#### Table III

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adenine nucleotides</th>
<th>ATP</th>
<th>ADP</th>
<th>ATP + ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$0.26 \pm 0.3^*$</td>
<td>$22 \pm 0.3^*$</td>
<td>$13.45 \pm 0.61^*$</td>
<td></td>
</tr>
</tbody>
</table>

* Group comparisons, one-tailed t test, $p < 0.001$.
* Group comparisons, one-tailed t test, $p < 0.01$.

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produced a difference in rate of transport (Fig. 5). The change in $V_{\text{max}}$ of ATPase rather than in $K_c$ is also consistent with accelerated transport consequent to the increase in adenine nucleotides. Other factors may of course affect ATPase activity. The content of Mg$^{2+}$ has been shown to be higher in mitochondria from glucagon-stimulated rats (27, 28) and this may also affect the activity of ATPase, as has been shown recently for mitochondria prepared from hepatomas (29).

The disparity in the amount of exchangeable adenine nucleotides present in mitochondria isolated from control and glucagon-treated rats has significant implications for research in the action of hormones on hepatic metabolism. There have been approximately 15 mitochondrial functions reported to be increased by acute treatment with hormones. These include carboxylation of pyruvate (4), oxidation of various substrates (4, 30), transport of pyruvate (31), proton gradient (28, 32), membrane potential (28), citrulline formation (26, 33), Ca$^{2+}$ transport (30), rate of swelling with valinomycin ATP and K$^{+}$ (27), Mg$^{2+}$ content (27, 28). K$^{+}$ content (28), ATP/ADP ratio (2, 26, 34), energetic functions of submitochondrial particles (14), transport of ATP (28, 29), Pi-ATP exchange (this paper), succinic dehydrogenase activity (35), and uncoupler-dependent ATPase (1, 2, this paper). A number of these effects can now be rationalized to be the consequence of a greater content of adenine nucleotides in mitochondria from glucagon-stimulated tissue. For example, the stimulation of carboxylation of pyruvate and the formation of citrulline may result, in part at least, from the increased ATP present in the mitochondria. The increased rate of transport made possible by the elevation of intramitochondrial exchangeable nucleotides may account in part for stimulation of the rate of State 3 respiration and oxidative phosphorylation. In this regard, a recent report has appeared supporting the concept of limitation of the rate of oxidative phosphorylation by adenine nucleotide transport (25). The accelerated rate of nucleotide transport would also explain the stimulation of Pi-ATP exchange, the ATP-driven swelling in the presence of valinomycin, and, as reported here, the uncoupler-dependent ATPase.

On the other hand, increases in nucleotide content do not explain the hormonal stimulation of succinic dehydrogenase and the rate of uncoupled respiration of mitochondria, functions thought to be independent of adenine nucleotides, nor does it explain the stimulation of several energetic functions of sub mitochondrial particles (e.g. see Table II) which are inverted and, therefore, permit unrestricted access of ATP to the ATPase complex when ATP is added to provide energy for reverse electron flow or energy-dependent transhydrogenase. Both of these functions are stimulated in submitochondrial particles prepared from hormone-stimulated mitochondria (14).

The mechanism by which the difference in exchangeable nucleotide content of the mitochondria develops is not understood. It is not the result of a difference in total adenine nucleotide content of the liver cells, as this has been shown to be unchanged by glucagon treatment (26, 34). The technique of Zuurendonk and Tager (36) which utilizes low concentrations of digitonin in the presence of atracyloside to separate mitochondrial and cytoplasmic cellular compartments rapidly is probably the best method currently available to measure mitochondrial components as they exist in the isolated hepatocyte. Three groups of workers (2, 26, 34) using this technique have reported the ATP and ADP content of the mitochondrial compartment of hepatocytes treated with glucagon. Two of the three reports indicate a mean increase of 17 to 20% in exchangeable nucleotides present after glucagon treatment (2, 34), suggesting that the effect is probably present in mitochondria in vivo. Furthermore, similar increases in mitochondrial nucleotides have been found after treatment with epinephrine (2, 37). The nucleotide differences may possibly be accentuated during preparation of the mitochondria, and further experiments will be required to ascertain whether the isolation procedure results in a loss of nucleotides in the control mitochondria or a gain by the glucagon-stimulated organelles or either. Mechanisms of net loss or gain of nucleotides have not been studied extensively. It has been reported (38, 39) that pyrophosphate will exchange for ATP and ADP leading to decreases or increases of mitochondrial nucleotides depending upon the direction of the exchange. The ability of rat liver mitochondria to synthesize pyrophosphate (40) provides a potential mechanism by which the total amount of intramitochondrial ATP and ADP could be increased by pyrophosphate-adenine nucleotide exchange. AMP within the mitochondria is also theoretically a reservoir or a sink for the exchangeable adenine nucleotides, ADP and ATP. However, the quantity of AMP present in control mitochondria (34) is not adequate to explain the increase in ATP + ADP in hormone-stimulated mitochondria even if all of the AMP were to be converted to the di- or trimucleotides.

Acknowledgments—The capable technical assistance of Mrs. Diane Brannock and Mr. Ralph Picking is gratefully acknowledged.

Note Added in Proof—Pp pic et al. (41) have reported that treatment of rats for 1 hour with glucagon increases the exchangeable adenine nucleotide (ATP + ADP) content of hepatic mitochondria by 70%.

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