Control of Ammonia Formation From ATP in a Multienzyme System of Liver in Presence of Uncouplers of Oxidative Phosphorylation

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SUMMARY

An *in vitro* system composed of isolated rat liver mitochondria and an AMP deaminase-containing cytoplasmic protein fraction has been employed for the study of the effect of uncouplers on the enzymic conversion of ATP to IMP + NH_4^+. Under these conditions one main pathway of ATP degradation is operative. It was shown that this metabolic sequence is initiated by activation of latent mitochondrial ATPase by uncouplers. Under uncoupled conditions, mitochondrial adenylate kinase converts ADP to AMP, which is almost quantitatively converted to IMP + NH_4^+ by cytoplasmic AMP deaminase. Oligomycin, which has no effect on AMP deaminase or adenylate kinase but counteracts the activating effect of uncouplers on mitochondrial ATPase, inhibits the ATP → IMP + NH_4^+ pathway by blocking the initiating step. An ATP-dependent activation of AMP-deaminase by Mg^{++} has been demonstrated. Relevance of these mechanisms to molecular control of cellular events is discussed.

INTRODUCTION

In the course of studies concerned with control mechanisms of glutamate metabolism operating in rat liver homogenates, it was observed that 2,4-dinitrophenol caused an accumulation of NH_4^+ (1). Quantitative analysis of amino acids revealed that oxidative deamination of glutamate could not account for excess NH_4^+ formation since this amino acid was quantitatively converted to aspartate (1, 2). Liver homogenates used for *in vitro* experiments were supplemented with added ATP to a level corresponding to a concentration of adenyl nucleotides found in intact liver (in the range of 2 to 3×10^{-3} M); therefore it was suspected that NH_4^+ might have originated from ATP.

The *in vitro* formation of NH_4^+ in presence of ATP and various tissue extracts is a well known phenomenon. However, a detailed multienzymic mechanism of this process in specific animal tissues functioning under various experimental conditions has not been clarified. The following considerations may illustrate this statement. Two enzymes, AMP deaminase (3) and adenosine deaminase (cf. 4) catalyze the formation of NH_4^+ from AMP or adenosine, respectively. Starting with ATP, at least two alternate routes of AMP formation exist, by way of ATPases and adenylate kinase, and by direct hydrolytic cleavage of ATP to AMP and pyrophosphate (5). Deamination of AMP may then occur directly, or hydrolytic cleavage of AMP to adenosine by 5′-nucleotidase (4) may precede deamination of adenosine by its specific deaminase. The question now arises whether in different tissues NH_4^+ formation from ATP is the result of simultaneous contribution of all possible metabolic sequences,

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or alternatively, if there are restrictive mechanisms which define the prevalence of one particular type of pathway.

It is clear that this question is meaningful only with respect to multienzymic control mechanisms. It was shown recently that various animal tissues exhibit apparently characteristic multienzymic patterns (6, 7), even though the enzymic composition of these tissues is similar. Metabolic specificity is manifested by preference for one among several possible multienzymic pathways.

There are indications that NH_4^+ formation from ATP may be controlled by feedback type mechanisms. It was found by Baer *et al.* that ATP has a powerful inhibitory effect on 5′-nucleotidase of heart tissue ($K_i = 1.83 \times 10^{-6}$, cf. 4); thus one of the enzymes capable of participating in the degradation of ATP is almost certainly blocked under physiological conditions. This control mechanism appears to operate also in liver tissue since only traces of adenosine could be detected in liver homogenates in presence of 2–3 mM ATP (cf. 1). Experiments described in this paper are specifically concerned with the control of the enzymic pathway initiated by uncouplers of oxidative phosphorylation leading from ATP to NH_4^+ and IMP. It is probable that identification of this pathway points to a metabolic effect of uncouplers which has not been appreciated as a part of their toxic action.

MATERIALS AND METHODS

Rat liver mitochondria were isolated (from livers of male Long-Evans rats of average body weight of 150–180 g) by differential centrifugation of homogenates, prepared in 0.25 *M* sucrose (containing 0.05% EDTA, pH 7.4) as described previously (1,2). Sedimented mitochondria were first washed with sucrose–EDTA solution, then a second time with 0.15 M KCl (containing no EDTA, pH 7.4). Mitochondria treated in this fashion were resuspended in 0.15 M KCl (containing no EDTA) for incubation experiments. Analytical procedures for protein and NH_4^+ (the latter by enzymic reoxidation of DPNH in presence of crystalline glutamic dehydrogenase by α-ketoglutarate and limiting amounts of NH_4^+) were previously described (cf. 1, 2). Rat liver AMP deaminase fraction was prepared from homogenates made up in 0.15 M KCl containing 0.05% EDTA (pH 7.4) centrifuged at 105,000 *g* for 1 hr. Since AMP deaminase activity of liver homogenates was present entirely in the supernatant fluid, this fluid was decanted and the deaminase-containing protein fraction separated (at 15°) by addition of a solution of 35% (w/v) of Na_2SO_4 sufficient to achieve a final concentration of 0.1 g Na_2SO_4/1 ml. The resulting precipitate was discarded, and after the Na_2SO_4 concentration was increased in the supernatant to 0.15 g/ml, AMP deaminase was quantitatively recovered in the resulting precipitate. This readily reproducible fractionation yielded about 10-fold concentration of enzyme activity compared to the crude high speed supernatant. The specific activity of the AMP-deaminase fraction varied between 0.1 and 0.3, using only AMP as substrate (expressed in μmoles of NH_4^+ formed by 1 mg protein/1 min at pH 7.4 and 29°, calculated from V_{max} values, see Fig. 2). For kinetic measurements, this protein fraction was dissolved in 0.05 M Tris (pH 7.4) containing 10^{-4}M GSH and 0.05% EDTA, and kept frozen at −15°. Enzyme activity remained constant for about 5 days. This protein fraction contained no adenylate kinase and only traces of ATPase activity (between 0.002 and 0.003 μmole of orthophosphate liberated from 10^{-2} M ATP/1 mg protein/1 min at pH 7.4 and 29° in presence of 10^{-3} M $MgCl_2$). Adenosine deaminase activity was not studied because adenosine was not formed from ATP under our experimental conditions (see Results, section A). Since present studies were primarily concerned with the elucidation of subcellular enzymic mechanisms, this crude deaminase fraction was employed in combination with mitochondrial systems and for preliminary kinetic experiments. Extensive purification and detailed kinetic analysis

of the deaminase is the subject of separate studies.

The multienzyme system employed for the determination of the metabolic pathway of ATP degradation was composed of liver mitochondria (10 mg protein) and cytoplasmic deaminase fraction (10 mg protein), suspended or dissolved in 3 ml reaction medium containing 10^{-2} M ATP, 10^{-3} M $MgCl_2$, 8.3×10^{-2} M Tris buffer, pH 7.4 and 0.15 M KCl (no EDTA). This system (containing mitochondria and deaminase alone or in combination) was incubated with mechanical shaking aerobically in Warburg vessels for 30 minutes at 30° in absence and presence of either 2,4-dinitrophenol (10^{-4} M), 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (4 μg/3 ml), and oligomycin (4 μg/3 ml), alone or in combination. In "controls" the reaction was stopped at 0 time (for blank values), and in "experimental" vessels at the end of incubation by 0.3 ml 30% $HClO_4$ (added in an ice bath). After removal of ClO_4^- as the K^+ salt, analyses for orthophosphate (P_i) (8), NH_4^+, ATP, ADP, AMP, and IMP were performed simultaneously by both chemical and enzymic methods (9–11). In evaluating analytical results, it is important to consider that the significance of numerical values must be weighed in terms of the accuracy of various analytical methods employed. Orthophosphate values ranging between 0.76 and 1.2 μmoles/3 ml reaction volume, as well as NH_4^+ between 0.05 and 0.2 μmoles/3 ml, approach the lower limits of sensitivity of analytical methods, including inaccuracies of dilutions; thus variations within these ranges have no experimental significance. The accuracy of analytical results above these levels is within ± 10%. The accuracy of nucleotide analyses also depends on their concentrations; below 0.5 μmole/3 ml, accuracy varies within ± 20%, while above this level it improves to ± 10%. The overall reliability of data is thus within an estimated ± 15 to 20%.

AMP deaminase activity was measured kinetically by coupling this enzymic reaction to glutamate dehydrogenase. In presence of a large excess of crystalline glutamate dehydrogenase, NH_4^+ formed by deamination instantly reoxidizes DPNH in presence of excess α-ketoglutarate; thus spectrophotometric monitoring of the disappearance of DPNH (at 340 mμ) provides a convenient kinetic enzyme assay which is equally sensitive to the direct spectrophotometric determination of AMP disappearance (or IMP formation). Coupling of deaminase with glutamate dehydrogenase is desirable also because at 340 mμ no serious spectral interference by UV light-absorbing tissue components or by AMP (~ 10^{-2} M) occurs. The only critical factor in this coupled deaminase assay is the presence of an excess amount of glutamate dehydrogenase, since the velocity of reductive amination of α-ketoglutarate must greatly exceed the rate of NH_4^+ formation, a condition easily established empirically (see Fig. 1). Spectrophotometric analyses, as well as kinetic measurements (at 29°), were carried out in a Zeiss PQ II spectrophotometer

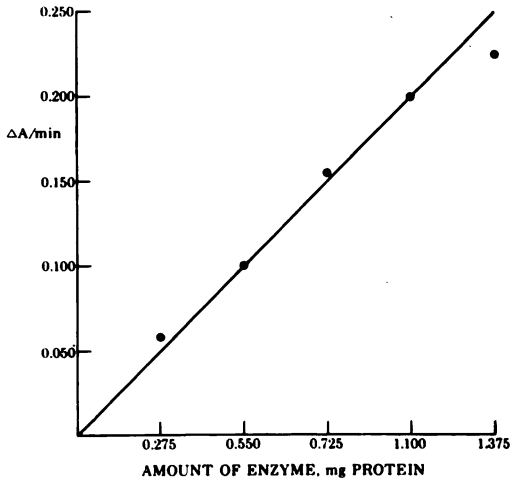

FIG. 1. *Coupled spectrophotometric assay*

The assay was made up as follows: 10^{-1} M KCl; 10^{-2} M AMP; 10^{-3} M $MgCl_2$; Tris-hydrochloride 0.1 M, pH 7.4; 10^{-4} M DPNH; 10^{-2} M α-ketoglutarate; 30 μl glutamic dehydrogenase (30 μg of crystalline enzyme protein). The reaction was started by the addition of varying amounts of cytoplasmic deaminase fraction (see Methods). Final volume = 1 ml; light path = 1 cm. Initial velocities were obtained by linear extrapolation from direct chart tracings. Temperature = 29°.

connected to a Honeywell chart recorder. In kinetic tests, initial rates of DPNH reoxidation (caused by enzymic NH_4^+ formation from AMP) were determined after traces of NH_4^+ present as unavoidable contamination of reagents were removed by glutamic dehydrogenase during preincubation for 4–5 minutes of the reaction system containing all components except the deaminase, which was added after preincubation at 0 time in a volume of 0.1 ml. Prior to addition of deaminase, DPNH content was readjusted to 10^{-4} M following the cessation of DPNH oxidation due to traces of NH_4^+ in reagents.

All reagents were of analytical grades; 2,4-dinitrophenol was recrystallized from ethanol–water, while 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (abbreviated TTFB) (refs. 12, 13) was a sample obtained from Dr. K. H. Büchel through the courtesy of Dr. Britton Chance (University of Pennsylvania, The Johnson Foundation, Department of Biophysics and Physical Biochemistry, Philadelphia, Pennsylvania). Oligomycin was a gift from Dr. Henry A. Lardy (University of Wisconsin, Institute for Enzyme Research, Madison, Wisconsin). Solutions of crystalline ammonium-free glutamate dehydrogenase (10 mg enzyme/ml) were obtained from Sigma Chemical Company and from Boehringer Company. 1,2-Diaminocyclohexanetetraacetic acid (cf. 19) was a gift from Dr. Jean Botts (University of California, Cardiovascular Research Institute, San Francisco, California).

RESULTS

A. *The Effect of Uncouplers on Combined Enzyme Systems of Rat Liver*

It was readily established that ATP is not a precursor of NH_4^+ when incubated with isolated subcellular fractions unless conditions for AMP formation are created. Among the various types of ATPases in liver, only the latent mitochondrial ATPase responds with a very large activation to uncouplers of oxidative phosphorylation (14), a finding that strongly suggests a possible rate-limiting role of mitochondrial ATPase in the proposed sequence of reactions (see Eq. 1). It has been found (cf. ref. 1) that 85% of ATP or ADP added to actively respiring liver homogenates is maintained as ATP for 30 min at 30°. Addition of 2,4-dinitrophenol (5×10^{-5} to 10^{-4} M) reduces the steady state level of ATP to about 30% of controls, and under these circumstances variable amounts of IMP and NH_4^+ accumulate. It is easily seen that, depending on the availability of substrates, even in the presence of uncouplers, part of ADP is reconverted to ATP by substrate-level phosphorylations, and part of NH_4^+ is consumed by various reamination reactions; thus stoichiometry (in terms of ATP, P_i NH_4^+, and IMP) capable of predicting a metabolic pathway can hardly be expected. The disturbing effect of interfering reactions was reduced to a minimum by the use of isolated liver mitochondria in combination with a cytoplasmic deaminase fraction (see Methods). This system maintains an endogenous respiration of 0.4 μmole O_2/10 mg mitochondrial protein/30 min at 30°. From results obtained with this system the mechanism shown in Eq. 1 was formulated.

$$\begin{aligned} 2\,ATP &\rightarrow 2\,ADP + 2\,P_i \\ 2\,ADP &\rightarrow ATP + AMP \\ AMP &\rightarrow IMP + NH_4^+ \end{aligned}$$

$$\text{Sum: } ATP = 2\,P_i + IMP + NH_4^+ \qquad (1)$$

It is evident from Table 1 that cytoplasmic deaminase fraction has only negligible ATPase activity (Table 1, No. 1). Mitochondrial ATPase (Table 1, No. 2) is not influenced by the cytoplasmic fraction; however, combination of both systems results in equimolar NH_4^+ and IMP accumulation (Table 1, No. 3). In this system, the sum of IMP and ADP formed seems to be about 36% less than the amount of ATP consumed (or P_i formed), indicating that even though excess deaminase is present, the system has not reached the condition described by Eq. 1. It is possible that the apparent disappearance of approximately 16% of ATP which could not be readily accounted for by experimental inaccuracies (± 20%) is due

TABLE 1

Changes in the concentrations of ATP, IMP, P_i, and NH_4^+ during aerobic incubation

The composition of the reaction system is described in the text (Results, Section A). Abbreviations: Cytopl. fr. = cytoplasmic deaminase fraction; Mito. = rat liver mitochondria; 2,4-DNP = 2,4-dinitrophenol (10^{-4} M); TTFB = 4,5,6,7-tetrachloro-2-trifluoromethyl benzimidazole (4.2×10^{-6} M); Oligo. = oligomycin (4 μg/3 ml); P_i = orthophosphate.

No.	Experimental conditions	$+\Delta P_i$	$+\Delta NH_4^+$	$-\Delta ATP$	$+\Delta ADP$	$+\Delta AMP$	$+\Delta IMP$
1	Cytopl. fr. alone	0.76	0.05	0.6	0.3	—	—
2	Mito. alone	2.3	0.06	2.8	2.0	0.05	—
3	Cytopl. fr. + Mito.	2.8	1.07	2.8	0.6	—	1.25
4	Cytopl. fr. + 2,4-DNP	0.80	0.10	0.6	0.3	—	—
5	Mito. + 2,4-DNP	14.4	0.16	13.0	7.0	6.0	—
6	Cytopl. fr. + Mito. + 2,4-DNP	24.2	11.9	12.3	1.3	0.05	10.9
7	Cytopl. fr. + TTFB	1.2	0.20	0.84	0.2	—	—
8	Mito. + TTFB	12.7	0.16	11.0	7.1	4.3	—
9	Cytopl. fr. + Mito. + TTFB	22.8	10.4	13.0	2.0	0.33	9.9
10	Cytopl. fr. + Oligo.	1.2	0.20	1.70	1.0	—	—
11	Mito. + Oligo.	1.6	0.05	1.80	1.5	0.03	—
12	Mito. + Cytopl. fr. + Oligo.	3.0	0.4	2.90	0.7	—	0.9
13	Cytopl. fr. + 2,4-DNP + Oligo.	0.83	0.11	0.20	0.4	—	—
14	Mito. + 2,4-DNP + Oligo.	4.2	0.13	5.90	3.2	0.7	—
15	Mito. + Cytopl. fr. + 2,4-DNP + Oligo.	6.2	2.7	4.80	1.1	—	2.5
16	Cytopl. fr. + TTFB + Oligo.	1.0	0.2	0.72	0.3	—	—
17	Mito. + TTFB + Oligo.	4.7	0.16	4.65	4.6	0.66	—
18	Mito. + Cytopl. fr. + TTFB + Oligo.	5.8	2.4	5.00	0.9	—	2.5

to accumulation of nucleotide derivatives not detected by our methods. This small discrepancy was noticed only when oxidative phosphorylation was intact. This steady-state condition[2] indicates that at least two systems compete for the ADP formed by the low "ATPase" activity of liver mitochondria. One is oxidative phosphorylation, including substrate-level phosphorylation, regenerating ATP and probably other unknown nucleotide derivatives by oxidation of endogenous substrates; the second is adenylate kinase, providing AMP, the substrate of the deaminase. Uncoupling agents have no effect on enzymic activities of the cytoplasmic deaminase fraction (Table 1, Nos. 4 and 7), but greatly increase ATPase activity of mitochondria (Table 1, Nos. 5 and 8). Activated ATPase provides an increased amount of substrate for adenylate kinase in mitochondria, resulting in an accumulation of ADP and AMP, the sum of which agrees well with the amount of ATP disappearance and P_i accumulation (Table 1, Nos. 5 and 8). When uncoupling agents are added to a mixture of mitochondria and cytoplasmic deaminase fraction (Table 1, Nos. 6 and 9), the stoichiometry of the steady-state concentration of reactants agrees with the one predicted by Eq. 1. The "initiating" role of activated mitochondrial ATPase on deamination was further tested by the use of oligomycin, which is known to inhibit the activating effect of uncouplers on mitochondrial ATPase (14, 15). Oligomycin has no effect on the very slow hydrolysis of

[2] This experimental condition (i.e., incubation for 30 min) has been previously defined as "pseudo-steady state" (cf. 2), which is a kinetically more correct expression. For the sake of simplicity, we refer to this condition here as "steady state."

ATP which occurs in presence of the cytoplasmic protein fraction (Table 1, Nos. 10 and 13). Apparent mitochondrial ATPase in absence of uncouplers is also uninfluenced by oligomycin (Table 1, Nos. 11 and 12), while 88% of the activating effect of 2,4-dinitrophenol is counteracted (Table 1, No. 14) by this substance. Quite similar effects were observed on the ATPase activation by TTFB (Table 1, No. 17). Deamination initiated by uncouplers in combined *in vitro* systems is inhibited by oligomycin even though the deaminase itself is insensitive to both oligomycin and uncouplers (Table 1, Nos. 16 and 18). These results clearly point to the operation of the sequential enzyme system yielding the balance described by Eq. 1. Adenylate kinase was not affected by either the uncouplers or oligomycin.

Hydrolytic cleavage of ATP to pyrophosphate and AMP (5) could provide an alternate pathway to furnish substrate for AMP deaminase from ATP. Measurable contribution of the pyrophosphate-forming enzyme in liver systems can be ruled out on the following grounds. (a) The pH optimum of this enzyme is between pH 8.4 and 8.8, and it has negligible activity at pH 7.4 (less than 0.1 μmole pyrophosphate formed in 210 min at 37° by 5 mg protein, cf. 11). (b) Pyrophosphate-forming enzyme has no Mg^{++} requirement, whereas the system described in this paper functions maximally in presence of added Mg^{++}. (c) Balance studies consistently revealed that ATP disappearance is always accounted for as P_i and ADP, and AMP formation occurs only as a consequence of the adenylate kinase reaction. (d) Uncouplers, which serve as activators of the enzymic sequence leading to NH_4^+ and IMP accumulation, are not known to affect the rate of pyrophosphate formation from ATP. A similar argument holds for oligomycin.

B. *Mechanism of Deamination of AMP by the Cytoplasmic Protein Fraction*

It is difficult to evaluate in kinetic terms the triggering mechanism of activation of "latent" mitochondrial ATPase because appearance of this enzymic activity is probably due to the interaction of a variety of presently unknown intramitochondrial components (cf. 16). However, the cytoplasmic AMP deaminase, even in crude form, lends itself to more decisive kinetic analyses. Results of preliminary kinetic experiments are as follows. The validity of the coupled AMP–deaminase–glutamate dehydrogenase assay is shown in Fig. 1. Linear relationship between initial rates and deaminase protein content exists. When $1/v$ vs. $1/S$ plots are drawn, instead of a linear relationship, a curve with upward trend emerges. However, when $1/v$ is plotted against $1/AMP^2$, a straight line is obtained. The simplest explanation for such a phenomenon is that deaminase may combine with 2 molecules of AMP, one at the substrate, the other at a "modifying" site. When ATP and AMP are present simultaneously, $1/v$ vs. $1/S$ plots (S referring to AMP only) are linear (Fig. 2).

While the apparent V_{max} is unaffected by ATP, apparent K_m for AMP is shifted by ATP from 7.3×10^{-3} M to 1.7×10^{-3} M (Table 2). This effect of ATP on the shape of the saturation curve for AMP with respect to the deaminase results in a large apparent increase of reaction velocity at low AMP concentrations, a situation likely to occur in cells and some subcellular systems. A more quantitative evaluation of the effect of ATP on the apparent K_m for AMP has not been carried out with the crude enzyme, but is the subject of further work. However, under conditions described in Table 2, 1 or 5×10^{-3} M ATP has almost the same effect on the apparent K_m of AMP, suggesting that ATP at low concentrations may be bound to the modifying site of the enzyme more tightly than AMP. In agreement with previous results of Mendicino and Muntz (17), ADP was found to have no effect on deaminase activity of the cytoplasmic protein fraction. Addition of ADP to systems containing adenylate kinase (e.g., in mitochondria) yields ATP + AMP, resulting in the same effect on deamination of AMP as addition of ATP itself. The effect of Mg^{++} and ATP

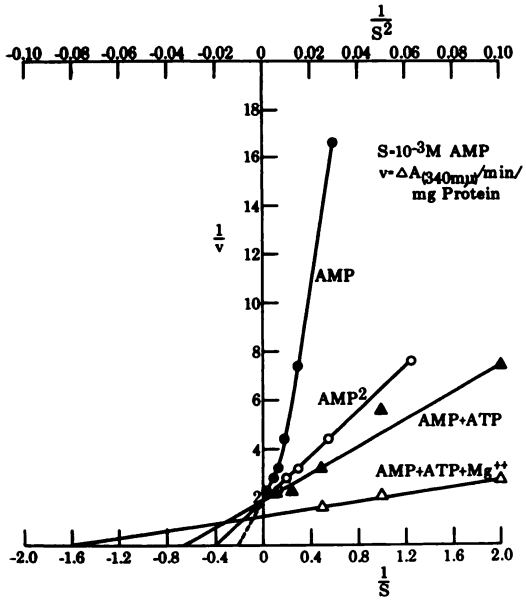

FIG. 2. *The effects of AMP concentration on rates of deamination in presence of ATP* (5×10^{-3} M), *$MgCl_2$* (10^{-3} M)

In the double reciprocal plot the lower abscissa expresses $1/S \times 10^{-3}$ M AMP, and the upper $1/S^2 \times 10^{-6}$ M AMP, the latter referring to the experimental line marked *AMP^2*. Conditions for kinetic measurements are described under Fig. 1. The amount of cytoplasmic protein fraction was 1 mg protein/ml.

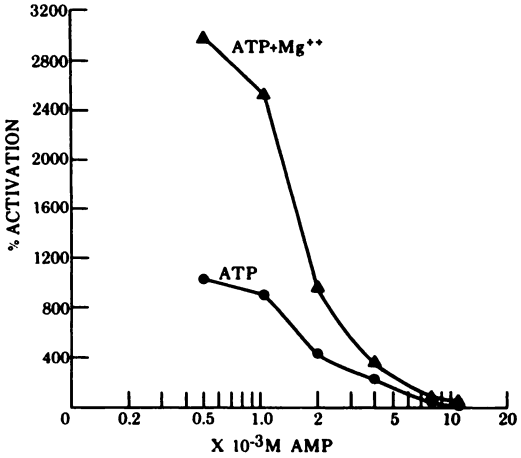

FIG. 3. *Activating effect of ATP* (5×10^{-3} M) *and $MgCl_2$* (10^{-3} M) *on initial velocity of deamination of AMP by cytoplasmic protein fraction in presence of varying concentrations of AMP*

Ordinate shows per cent increase in velocity as compared to a system containing only AMP. Abscissa shows concentration of AMP on a logarithmic scale. For assay conditions see legend to Fig. 2.

AMP to 0.6×10^{-3} M (Table 2, No. 3). The mechanism of this phenomenon needs to be studied further with purified deaminase. The role of Mg^{++} was further studied with the aid of a powerful Mg^{++} chelating compound, 1,2-diaminocyclohexanetetraacetic acid (18). This chelating agent completely abolished the activation by Mg^{++}, but did not alter the effect of ATP or influence the rate of deamination of AMP in the absence of ATP. Assuming that AMP deaminase is a metallo enzyme, the binding of the metal by the protein could be sufficiently stable to exclude the

TABLE 2

Apparent kinetic constants for crude AMP deaminase

Apparent kinetic constants were calculated from initial velocity measurements by the coupled deaminase procedure (see legend to Fig. 1).

No.	Experimental conditions	K_m (approx)	V_{max} (approx)
1	AMP (± Mg^{++})	7.3×10^{-3}	0.550
2	AMP + ATP (5×10^{-3} M)	1.7×10^{-3}	0.550
3	AMP + ATP (5×10^{-3} M) + Mg^{++} (2×10^{-3} M)	0.6×10^{-3}	0.832

on the rate of deamination of AMP is quite unusual; Mg^{++} reinforces the activating effect of ATP, and this effect is largest at low AMP levels (Fig. 3). When AMP alone is the substrate, Mg^{++} has no effect (Fig. 4). In contrast to ATP alone, which does not change V_{max}, Mg^{++} plus ATP increases apparent V_{max} by approximately 30% and diminishes the apparent K_m for

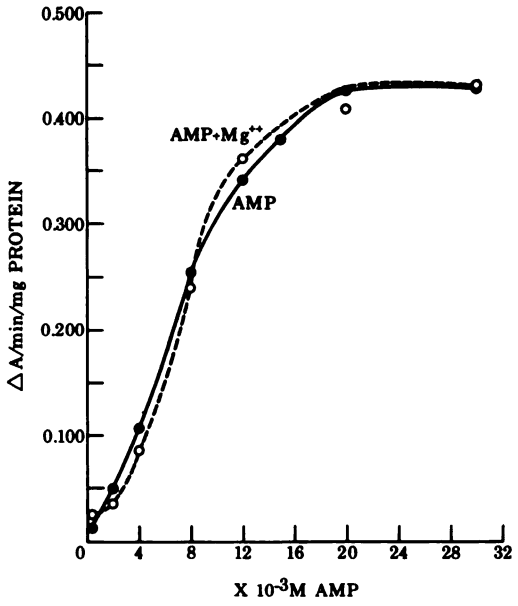

FIG. 4. *The effect of $MgCl_2$ (10^{-3} M) on the rate of deamination at various concentrations of AMP*

For assay conditions see legend to Fig. 1.

interaction of an added chelating agent with an enzyme-bound metal. The effect of externally added Mg^{++}, on the other hand, can still be effectively removed by the Mg^{++} chelating agent. Added Mn^{++} is equally effective as Mg^{++}, while Ca^{++} has no influence. Whether or not externally added Mg^{++} exerts its effect by interaction with ATP (and the enzyme) can only be determined with highly purified AMP deaminase.

It should be mentioned that none of these factors (ATP, AMP, Mg^{++}, Ca^{++}, 1,2-diaminocyclohexanetetraacetic acid) influenced the reagent role of the large amount of crystalline glutamate dehydrogenase employed in the coupled deaminase assay. The rate of removal of enzymically formed NH_4^+ by amounts of glutamate dehydrogenase employed is immeasurably rapid; thus the presence of modifiers of glutamate dehydrogenase which affect this enzyme when present at very low concentrations (approxnmately 10^{-9} M) (19) remains undetectable.

DISCUSSION

Rapid degradation of adenyl nucleotides to IMP and NH_4^+ may be regarded as a metabolic consequence of uncoupling of oxidative phosphorylation. There are several control points in this pathway which under energy-coupled conditions render it largely inoperative. The first is the latent state of mitochondrial ATPase. Experimental results of Swanson (20), Myers and Slater (21), and more recently of Fonyo *et al.* (22) support the view that activation of latent ATPase, at least by OH^-, is a reversible process; thus physiological mechanisms may exist which can balance the activity of this enzyme. The inhibition of the uncoupler-initiated degradative path of ATP to $NH_4^+ +$ IMP by oligomycin provides another molecular mechanism demonstrating the reversibility of this triggering step in pharmacological terms. It is obvious that uncouplers must exert a more complex effect than OH^- since it is known that, e.g., the ATP-ADP exchange reaction itself is also 2,4-dinitrophenol sensitive (cf. 16). Another control zone of this pathway is the efficiency of glycolytic and substrate-level ATP synthesizing reactions, which may temporarily compensate the effect of uncouplers. As soon as oxidative phosphorylation is uncoupled and other much less efficient ATP-synthesizing mechanisms cannot compensate any more for a loss of ATP (e.g., because of exhaustion of substrates), the second or rapid phase of the ATP $\rightarrow$ IMP $+ NH_4^+$ path becomes operative. Through the action of adenylate kinase (myokinase), AMP is made available to AMP deaminase. This enzyme responds to the activating effect of ATP reinforced by Mg^{++} with a large increase in "catalytic efficiency," as measured by a drastic decrease of apparent K_m for AMP and an increase in the velocity of enzymic catalysis (by Mg^{++}). In a brief note Setlow *et al.* (23) concluded that ATP activation of AMP-deaminase represents a self-controlling circuit of guanine nucleotide formation whereby GTP serves as a feedback inhibitor of AMP deaminase by

competing with ATP as an activator. This proposed scheme in terms of enzymic reactions is entirely feasible, but its metabolic significance depends on the rate of intracellular AMP formation. Added adenine nucleotides in actively respiring coupled systems tend to be maintained as such (predominantly as ATP, cf. 1); thus special conditions must be created for significant conversion to AMP. Uncoupling by certain reagents provides such a metabolic signal. It is of interest to relate our results to metabolic effects of 2,4-dinitrophenol observed in other biological systems. Recently Simon *et al.* (24) described apparently selective inhibition of ribosomal RNA synthesis by 2,4-dinitrophenol in *Escherichia coli*. Whether or not a 2,4-dinitrophenol-activated breakdown of ATP along the pathway described contributes to the mode of action of 2,4-dinitrophenol on *E. coli* cannot be assessed from available experimental results. It was recently reported that administration of 2,4-dinitrophenol to guinea pigs results in a large decrease of total adenine nucleotide content of the liver (25). These results suggest that the enzymic mechanisms described in this paper are likely to operate *in vivo*. Preliminary experiments in our laboratory dealing with mechanisms of enzyme induction in rat tissues revealed that administration of 2,4-dinitrophenol (10 mg/kg injected intraperitoneally) to male Long-Evans rats (150–200 g) caused a 40% reduction of alanine transaminase in liver cytoplasm. However, the enzyme-inducing effect of prednisolone (2 mg per day for 4 days, injected intraperitoneally) was not inhibited, but rather significantly reinforced by simultaneous injection of 2,4-dinitrophenol.[3] These preliminary results suggest that *in vivo* metabolic effects of uncouplers cannot be explained merely by a rather general "hypermetabolism." It seems probable that a hitherto unsuspected diminution of adenine nucleotide pools is initiated by uncouplers. Through this mechanism a variety of effects on control of enzyme biosynthesis is possible with important consequences in other areas of cellular functions (e.g., mitosis).

[3] J. Aitchison, unpublished experiments.

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