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THE PHOSPHORYLATION OF INTRAMITOCHONDRIAL AMP: A SUGGESTION FOR THE COMPARTMENTATION OF ENDOGENOUS P_i POOL

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SUMMARY

1. The mitochondrial level of AMP gradually diminishes during incubation of mitochondria with glutamate but does not with succinate. This decline of AMP, associated with stoichiometric increase in ADP and/or ATP, is accelerated by the addition of electron acceptors or 2,4-dinitrophenol, while arsenite, arsenate and rotenone are inhibitory. These results are in agreement with the view that AMP is phosphorylated to ADP in the inner space of rat liver mitochondria *via* succinyl-CoA synthetase (succinate:CoA ligase (GDP), EC 6.2.1.4) and GTP:AMP phosphotransferase dependent on the oxidation of 2-oxoglutarate, which is promoted by the transfer of electron from NADH to the respiratory chain.

2. Studies of the periodical changes of chemical quantities of adenine nucleotides as well as of their labelling with $^{32}P_i$ reveals the following characteristics concerning mitochondrial phosphorylation. (i) In contrast to the mass action ratio of ATP to ADP, the ratio of ADP to AMP is not affected by the intramitochondrial concentration of P_i. (ii) $^{32}P_i$, externally added, is incorporated into ADP much more slowly than into γ-phosphate of ATP. (iii) Conversely, ATP loses its radioactivity from γ-phosphate position more rapidly than [^{32}P]ADP when ^{32}P-labelled mitochondria are incubated with non-radioactive P_i.

3. In order to elucidate the above characteristic properties of phosphorylation, a hypothetical scheme is proposed which postulates the two separate compartments in the intramitochondrial pool of P_i; one readily communicates with external P_i and is utilized for the phosphorylation of ADP in oxidative phosphorylation, while the other less readily communicates with external P_i and serves as the precursor of ADP *via* succinyl-CoA synthetase and GTP:AMP phosphotransferase.

INTRODUCTION

The phosphorylation of AMP instead of ADP is the event that takes place during the course of the substrate-level phosphorylation coupled to the oxidation of 2-oxoglutarate[1,2]. This conclusion was drawn first by Heldt and Schwalbach[1] based on the results of tracer experiments in which the incorporation of $^{32}P_i$ into the phosphorylation products was followed when the dismutation of 2-oxoglutarate

was allowed to proceed under anaerobic conditions. Generally speaking, however, the rate of incorporation of a radioactive precursor into a product reflects not only the net conversion rate, but also the activity of an exchange reaction or a change in the specific radioactivity of the labelled precursor. In this respect, therefore, the findings[2] that chemical quantity of mitochondrial AMP actually decreased as $^{32}P_i$ was incorporated into ADP appear to offer opportunities for providing a more direct insight into the phosphorylation reactions occurring in mitochondria.

In the present study, the feature of the phosphorylation of AMP, distinct from the phosphorylation of ADP, is revealed by following the periodical changes of mitochondrial content of ATP, ADP and AMP concurrently with the incorporation of $^{32}P_i$ into these adenine nucleotide fractions. The results obtained appear to be explainable by postulating the two compartments in mitochondrial P_i pool each of which supports the phosphorylation of ADP and AMP separately.

MATERIALS AND METHODS

Mitochondria isolated from rat liver according to Johnson and Lardy[3] were incubated as described previously[2]. The reaction mixture consists of 145 mM KCl, 1 mM EDTA, 20 mM Tris–HCl (pH 7.4), 14–55 mM sucrose (derived from mitochondrial suspension) and mitochondria in a total volume of 1.2 to 36 ml as indicated in tables and figures. Separation of nucleotides labelled during incubation was carried out on the thin-layer plate of polyethyleneimine–cellulose[4] after treatment of the acidified medium with charcoal. Chemical quantities of adenine nucleotides were measured enzymatically in Gilford Model 2400 spectrophotometer. The details of these and other procedures as well as sources of special reagents were the same as those in the preceding paper[2]. Atractyloside was a kind gift from Dr A. Bruni, University of Padova, Italy.

RESULTS

Relation of the phosphorylation of AMP to the substrate-level phosphorylation

A possibility that the disappearance of AMP observed during incubation of rat liver mitochondria, like the $^{32}P_i$ incorporation into ADP, is connected with the oxidation of 2-oxoglutarate was first examined by studying the effect of inhibitors on the mitochondrial level of AMP. The results are presented in Table I. In accord with our conclusion[2], based on tracer experiments, that ADP is formed *via* GTP: AMP phosphotransferase, which is present exclusively inside the adenine nucleotide barrier, atractyloside was without effect on the phosphorylation of AMP. In contrast, both arsenate, uncoupler of phosphorylation, and arsenite, the inhibitor of 2-oxoglutarate oxidation, were very effective in causing an accumulation of AMP (Expt. 1). In Expt. 2, mitochondrial suspension was incubated with 2-oxoglutarate, malonate and rotenone. Under these conditions, 2-oxoglutarate was oxidized only very slowly because regeneration of NAD through transfer of electrons to the respiratory chain was blocked by rotenone. This caused an accumulation of AMP. (Malonate was added to facilitate the entry of 2-oxoglutarate into mitochondria[5,6].) When the reaction mixture was further added with NH_3 which caused a dismutation of 2-oxoglutarate independent of the activity of respiratory chain, the mitochondrial

TABLE I

MITOCHONDRIAL CONTENT OF ATP, ADP AND AMP AT THE EQUILIBRIUM POSITION OF THEIR INTERCONVERSION AS AFFECTED BY INHIBITORS AND UNCOUPLERS OF THE SUBSTRATE-LEVEL PHOSPHORYLATION

Mitochondria from 600 mg rat liver were incubated in 6 ml reaction mixture for 3 min in the presence of additions as indicated and analyzed for ATP, ADP and AMP.

Expt	*Additions*	*Mitochondrial content of (nmoles/g liver)*		
		ATP	*ADP*	*AMP*
1	*Incubated with 10 mM glutamate and 10 mM $K^3Fe(CN)_6$*			
	None	144	143	22
	Atractyloside (0.05 mM)	142	133	22
	Arsenite (1 mM)	39	120	150
	Arsenate (1 mM)	40	106	156
2	*Incubated with 10 mM 2-oxoglutarate, 10 mM malonate and 0.2 μM rotenone*			
	None	20	261	101
	NH_4Cl(5 mM)	36	279	60
	NH_4Cl, arsenite	26	211	143

level of AMP decreased from 101 to 60 nmoles per g liver. This phosphorylation of AMP was again inhibited by arsenite. These results are in good agreement with the view that the phosphorylation of AMP takes place in rat liver mitochondria as a result of the substrate-level phosphorylation coupled to the oxidation of 2-oxoglutarate.

The effect of 2,4-dinitrophenol on the intramitochondrial adenine nucleotides is shown in Fig. 1, where mitochondria were incubated with or without glutamate as respiratory substrate in the presence of 0.1 mM P_i. A comparison of Figs 1A and 1C reveals that glutamate is required for the conversion of AMP to ADP (and then to ATP). As expected, mitochondrial ATP fell precipitously upon the addition of 2,4-dinitrophenol giving rise to a simultaneous increase of ADP (Figs 1B and 1D). In keeping with our recent observation[2] that 2,4-dinitrophenol is very effective in enhancing $^{32}P_i$ labelling of ADP, the addition of 2,4-dinitrophenol accelerated the conversion of AMP to ADP, notably in the presence of glutamate. A possible involvement of adenylate kinase in the 2,4-dinitrophenol-induced disappearance of AMP is safely excluded because the combined function of 2,4-dinitrophenol-induced ATPase and adenylate kinase should have caused a breakdown of ADP to AMP instead of the phosphorylation of AMP. The 2,4-dinitrophenol-induced conversion of AMP to ADP was blocked upon replacing glutamate by succinate (open symbols at 150 s in Fig. 1D) or upon inhibiting electron transport by rotenone (half-solid symbols), confirming our previous conclusion[2], based on tracer experiments, that the phosphorylation of AMP, distinct in nature from the phosphorylation of ADP, occurs dependent on the oxidation of glutamate which in turn is promoted by uncouplers through the accelerated transfer of electrons along the respiratory chain.

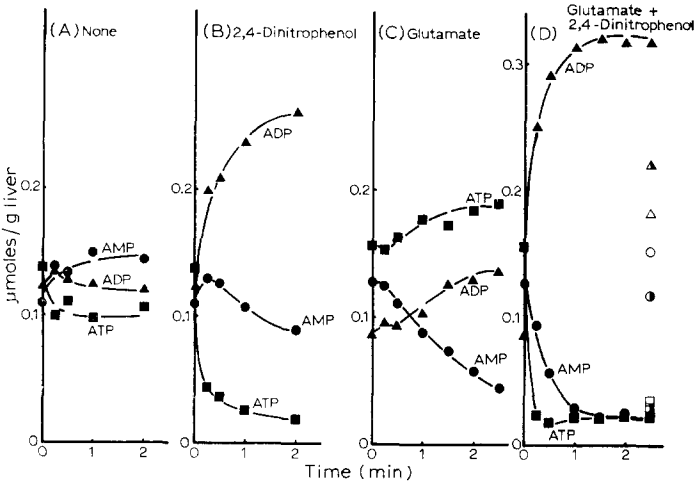


Fig. 1. Effect of 2,4-dinitrophenol on the mitochondrial level of adenine nucleotides. Mitochondria were incubated in 6 ml of the standard medium added with 0.1 mM P_i at a concentration of 100 mg liver equivalent per ml. Further additions are: Panel A, none; Panel B, 0.02 mM 2,4-dinitrophenol; Panel C, 10 mM glutamate; Panel D, 2,4-dinitrophenol and glutamate. Time course of ATP (■), ADP(▲) and AMP(●) are presented in each panel. In Panel D, ATP(□), ADP(△) and AMP(○) were estimated after 150 s incubation with 10 mM succinate instead of glutamate. The effect of rotenone on ATP(◨), ADP(◭) and AMP(◑) are also recorded.

Differential role of P_i in the phosphorylations of AMP and ADP

One of the most striking differences so far observed by us between the phosphorylations of AMP and ADP is related to the effect of P_i as summarized in Table II. Based on the finding that the mitochondrial content of adenine nucleotides almost reaches their equilibrium position in 2.5 to 3 min under our conditions (Figs 1–3, see also ref. 2), the ratio of ATP to ADP and the ratio of ADP to AMP were calculated as a measure of the degree of phosphorylation of ADP and AMP, respectively, after 3 min incubation under various conditions. It is seen in Table II that the addition of 0.1 mM P_i was very effective in raising the ATP/ADP ratio regardless of the concentration of mitochondrial protein and respiratory state. But 0.01 mM P_i was without effect in this respect. This P_i-induced increase in ATP/ADP ratio very likely results from the maintenance of phosphate potential[7] in the inner space of mitochondria, since the addition of 0.1 mM P_i did, but 0.01 mM P_i did not, cause an expansion of intramitochondrial P_i pool (Table III). In contrast, the ratio of ADP to AMP remained surprisingly unaffected by the addition of 0.1 mM P_i, suggesting that the phosphorylation of AMP is not directly dependent on the availability of P_i in mitochondria.

Thus, the presence of external P_i is indispensable for an optimal phosphorylation of the endogenous ADP. A typical time course of such an optimal phosphorylation of the endogenous adenine nucleotides is presented in Fig. 2 which shows that, after an initial breakdown of ATP to ADP, ATP is gradually formed at the expense of AMP with an ADP level kept essentially unchanged. This pattern is in good agreement with the previous report[8].

An additional factor, which differentially influences the phosphorylations

of ADP and AMP, was the concentration of mitochondria in the reaction mixture. Comparison of the phosphorylations occurring in the diluted (25 mg liver-equivalent/ml, Table II), in the concentrated (500 mg/ml, Table II) and in the intermediate (100 mg/ml, most experiments other than those in Table II) mitochondrial suspensions revealed that the higher ATP/ADP ratio was attained in the more con-

TABLE II

MITOCHONDRIAL LEVELS OF ATP, ADP AND AMP AS AFFECTED BY THE CONCENTRATION OF MITOCHONDRIA AND THE ADDITION OF P_i

Rat liver mitochondria were incubated for 3 min. Diluted: mitochondria from 720 mg liver incubated in 36 ml of medium, Concentrated: mitochondria from 600 mg liver in 1.2 ml of medium.

Additions	*P_i (0.1 mM)*	*Mitochondrial content (nmoles/g liver)*			*Ratio*	
		ATP	*ADP*	*AMP*	$\frac{ATP}{ADP}$	$\frac{ADP}{AMP}$
Diluted						
None	−	25	180	140	0.14	1.25
	+	61	161	122	0.38	1.32
Glutamate (10 mM)	−	27	227	86	0.12	2.63
	+	95	177	73	0.54	2.42
	+*	29	230	87	0.13	2.64
Glutamate, $K_3Fe(CN)_6$	−	33	247	70	0.13	3.53
	+	98	193	57	0.51	3.38
Concentrated						
None	−	120	122	87	0.98	1.40
	+	132	121	85	1.09	1.42
Glutamate, $K_3Fe(CN)_6$	−	177	135	28	1.31	4.82
	+	224	104	21	2.15	4.95

* 0.01 mM P_i added.

TABLE III

THE AMOUNT OF P_i IN THE INNER SPACE OF RAT LIVER MITOCHONDRIA

Mitochondria from 120 mg rat liver were suspended in 6 ml of the incubation medium. Immediately before or after incubation with the addition as indicated, the mitochondrial suspension was rapidly filtered and washed on the Millipore filter as described previously[2]. The filter was then extracted and analyzed for P_i according to the method of Itaya and Ui[18]. Means of two observations are presented.

nmoles/g liver

Before incubation	*After incubation with*			
	None	*2,4-dinitrophenol (0.05 mM)*	*P_i (0.01 mM)*	*P_i (0.1 mM)*
143	144	83	143	228

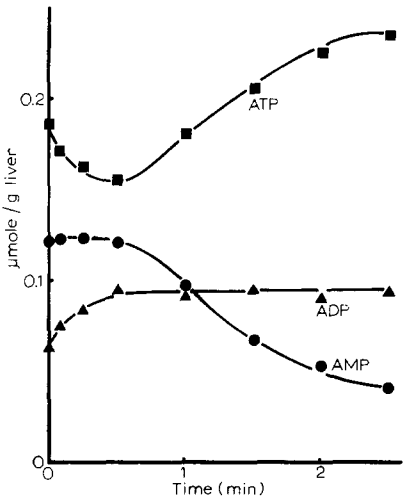


Fig. 2. Periodical changes of the mitochondrial level of ATP, ADP and AMP under the condition favorable for phosphorylation reactions. Mitochondrial preparation prepared from 600 mg liver were incubated in 6 ml of the reaction mixture containing 10 mM glutamate, 10 mM $K_3Fe(CN)_6$ and 0.1 mM P_i. ■, ATP; ▲, ADP; ●, AMP.

centrated solution and that, in contrast, only a slight change in ADP/AMP ratio was induced by changing the mitochondrial concentration. This again shows that the phosphorylation of AMP is distinct in nature from the oxidative phosphorylation.

Delayed $^{32}P_i$ labelling of ADP suggesting a compartmentalized pool of P_i in mitochondria

When fresh mitochondria are incubated, an abrupt breakdown of ATP due to a dilution of mitochondrial suspension tends to obscure the initial stage of phosphorylation (*e.g.* Fig. 2, see also Fig. 1 in ref. 2). This difficulty is overcome by using the mitochondrial preparation which has been depleted of ATP by means of preincubation with cyanide. Cyanide-induced inhibition of respiration can be then released by adding electron acceptors such as $K_3Fe(CN)_6$. Fig. 3 shows a typical time course of phosphorylation and $^{32}P_i$ incorporation in such a mitochondrial preparation with further addition of glutamate and 0.1 mM $^{32}P_i$. ADP which had accumulated during preincuabtion with CN^- was rapidly phosphorylated to ATP during the initial incubation time up to 5 s. This rapid phosphorylation of the endogenous ADP ("initial burst") is in keeping with Heldt *et al.*[9,10] and Duée and Vignais[11] who clearly indicated that internal ADP is phosphorylated very rapidly prior to external ADP. Following the "initial burst", ATP was generated more slowly but steadily at the expense of the endogenous AMP. In contrast, the level of ADP was kept fairly constant from 30 s to the end of incuabtion.

$^{32}P_i$ was incorporated into γ-phosphate of ATP very sharply, while the ADP fraction was labelled more slowly with an initial lag phase (Fig. 3B). Longer than 2 min incubation was required for the specific radioactivity of the ADP pool to attain its maximum level. which is roughly the same as the specific radioactivity of γ-[^{32}P]phosphate of ATP (Fig. 3C). The lower specific radioactivity of ADP

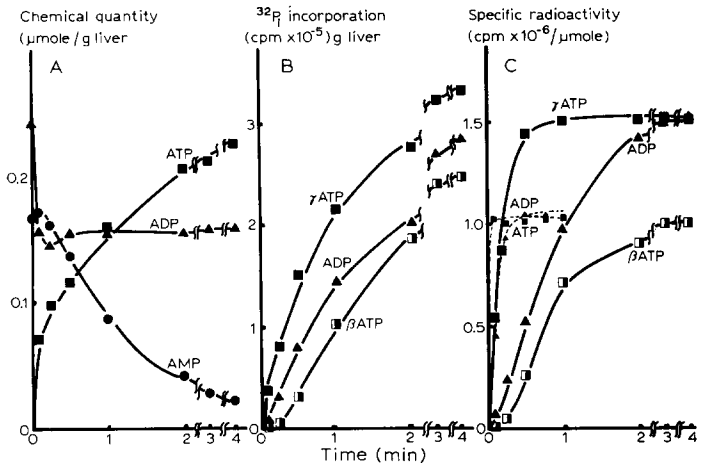


Fig. 3. Periodical changes of ATP, ADP and AMP and their ^{32}P content during incubation of the mitochondrial preparation pretreated with cyanide. Mitochondria isolated from 1.2 g rat liver were incubated in the reaction mixture containing 0.33 M sucrose, 20 mM Tris–HCl, 0.1 mM EDTA and 1 mM neutralized KCN in a total volume of 1.0 ml for 150 s at 25 °C. These cyamide-treated mitocondria were incubated after dilution as in Fig. 2 with further addition of 10 mM glutamate, 10 mM $K_3Fe(CN)_6$ and 0.1 mM P_i. [^{3}H]ADP in a tracer amount was also added. Each point represents the mean of two observations. Panel A: chemical quantities of ATP (■), ADP (▲) and AMP (●). Panel B: $^{32}P_i$ incorporation into γ-phosphate of ATP (■), β-phosphate of ATP (□) and ADP (▲). Panel C: the specific radioactivity of γ-phosphate of ATP (■—■), β-phosphate of ATP (□—□) and ADP (▲—▲) with respect to ^{32}P and of ATP (■---■) and ADP (▲---▲) with respect to ^{3}H.

than that of γ-phosphate of ATP at the initial phase cannot be fully explained by the delayed phosphorylation of AMP, because the specific radioactivity of ADP remains still lower at 1 min while the phosphorylation of AMP, as active as the phosphorylation of ADP, starts at 5 to 15 s (Fig. 3A). In order to clarify this apparent discrepancy between the phosphorylation of AMP and ^{32}P incorporation into ADP, the following experiment was undertaken; the result is presented in Fig. 4.

In the experiments in Fig. 4, $^{32}P_i$ was added at 15 s incubation time when the "initial burst" had ended and the steady increase of ATP at the expense of AMP had started. The original point on the abscissa in Figs 4A–4C is the time of this $^{32}P_i$ addition. Fig. 4A clearly shows that the phosphorylation of AMP initially tends to proceed prior to the incorporation of $^{32}P_i$ into ADP. This is in accord with a view that the endogenous pool of P_i, serving as the precursor of ADP, is slowly labelled with externally added $^{32}P_i$. In contrast, $^{32}P_i$ was incorporated into ATP at a very high rate relative to the generation of ATP at the initial stage of incubation (Fig. 4B), suggesting that the endogenous P_i pool is labelled with external $^{32}P_i$ very rapidly in this case. A possible explanation for this discrepancy between $^{32}P_i$ labelling of ATP and ADP might reside in the fact that $^{32}P_i$–ATP exchange reaction, which does not necessarily reflect the net phosphorylation of ADP, occurs at a higher rate than the $^{32}P_i$–ADP exchange reaction. This possibility was checked by comparing the incorporation of [^{3}H]ADP into ATP with its incorporation into AMP. The results are shown by the traces with dotted lines in

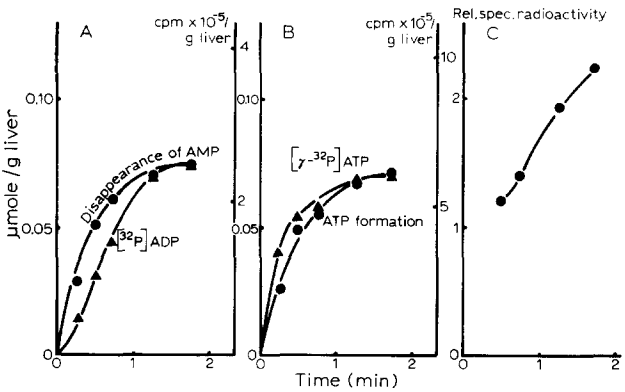


Fig. 4. The incorporation of $^{32}P_i$ into ADP and ATP as related to the disappearance of AMP and the production of ATP, respectively. Incubation of mitochondria was conducted in the same manner as in Fig. 3 except for the addition of carrier-free $^{32}P_i$ at 15 s, instead of 0 s, of the second incubation. 0 point on abscissa in each panel is the time of this $^{32}P_i$ addition. In Panel A, disappearance of AMP (●) starting at the time of $^{32}P_i$ addition is recorded, together with the incorporation of $^{32}P_i$ into ADP fraction (▲). In Panel B the increase of ATP (●) after the time of $^{32}P_i$ addition is plotted along with the incorporation of $^{32}P_i$ into γ-phosphate of ATP (▲). The specific radioactivity of P_i which serves as the substrate of AMP phosphorylation was calculated by dividing the ^{32}P content of ADP and of β-position of ATP at any time of incubation by the amount of AMP that had disappeared during the period up to that time. The ratio of this value to the specific radioactivity of ATP with respect to its γ-position is recorded in Panel C.

Fig. 3C. Indeed, the [^{3}H]ADP–ATP exchange reaction occurs more rapidly than [^{3}H]ADP–AMP exchange up to 10 s. But the specific radioactivity of [^{3}H]AMP became the same as that of [^{3}H]ATP as early as 15 s. It appears, therefore, that the delayed incorporation of $^{32}P_i$ into ADP relative to the incorporation into γ-phosphate of ATP cannot be accounted for in terms of lower rate of exchange reaction.

We were then led to a conclusion that the phosphorylation of AMP is supported by an endogenous pool of P_i to which external P_i is less available than to another P_i pool selectively serving as the substrate of the phosphorylation of ADP. We tentatively calculated the specific radioactivity of the P_i pool, which serves as a precursor of ADP, by means of dividing the cpm of $^{32}P_i$ incorporated into ADP and β-phosphate of ATP by the amount of AMP that has disappeared during the same incubation time. This tentative calculation is based on an assumption that the backward reaction from ADP to AMP does not take place. Since this assumption is unlikely in a strict sense, the calculated value may represent the upper limit of the real specific radioactivity. The calculated specific radioactivity relative to the specific radioactivity of γ-phosphate of ATP is plotted as a function of incubation time in Fig. 4C, which shows a gradual increase, in accord with the above view that the pool of P_i serving as the substrate of AMP phosphorylation is labelled with external $^{32}P_i$ more slowly than the P_i pool which is utilized directly for the phosphorylation of ADP.

When ^{32}P-preloaded mitochondria were exposed to nonradioactive P_i (Fig. 5), the specific radioactivity of ATP with respect to its γ-position declined rapidly,

while that of ADP showed a much slower decrease, indicating that γ-phosphate of ATP exchanges with external P_i more rapidly than ADP does. This observation is compatible with the afore-mentioned concept of two separate compartments concerning the intramitochondrial P_i pool differentially communicating with external P_i.

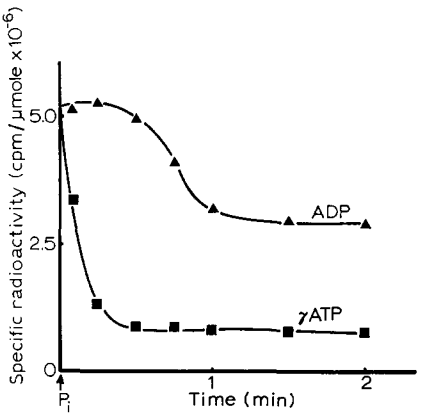


Fig. 5. The decline of ^{32}P content of γ-phosphate of ATP and ADP during incubation with non-radioactive P_i. ^{32}P-labelled mitochondria were prepared by incubating fresh mitochondria with carrier-free $^{32}P_i$ for 5 min. Other conditions are the same as in the case of pretreatment of mitochondria with cyanide in Fig. 3. Further incubation was conducted as in Fig. 3 with 0.2 mM P_i. ▲, specific radioactivity of [^{32}P]ADP; ■, specific radioactivity of [^{32}P]ATP with respect to γ-position.

Availability of the ADP generated from AMP as a phosphate acceptor for oxidative phosphorylation

The generation of [β-^{32}P]ATP as observed in Fig. 3 indicates that the ADP resulting from the phosphorylation of AMP can serve as a phosphate acceptor in the respiration-linked phosphorylation. In a quantitative sense, it is worthy of note here that the specific radioactivity of [β-^{32}P]ATP was maintained at a much lower level than that of [^{32}P]ADP; it might suggest that the newly formed ADP (from AMP) serves as the phosphate acceptor only to a lesser extent than the originally present ADP. This is only presumptive, however, because the lower specific radioactivity of [β-^{32}P]ATP could be explained alternatively by the fact that an enormous amount of ATP has accumulated prior to the onset of the ^{32}P incorporation into β-phosphate of ATP in Fig. 3. For the purpose of examining more closely the role of the ADP molecules originating from AMP as a phosphate acceptor of oxidative phosphorylation, the mitochondrial suspension was incubated with $^{32}P_i$ in the presence of 2,4-dinitrophenol to give rise to a labelling of the ADP fraction without yielding ATP. The results are presented in Fig. 6. In keeping with Fig. 1, the incubation with 2,4-dinitrophenol caused a rapid breakdown of ATP to ADP followed by a steadily phosphorylation of AMP to ADP (Fig. 6A). This phosphorylation of AMP results in a significant labelling of ADP after 2.5 min as shown in the initial point in Fig. 6B. Then, the phosphorylation of ADP to ATP was initiated by the addition of 0.3% albumin as shown in Fig. 6A. It is seen in Fig. 6B that $^{32}P_i$ was incorporated into β-phosphate as well as γ-phosphate

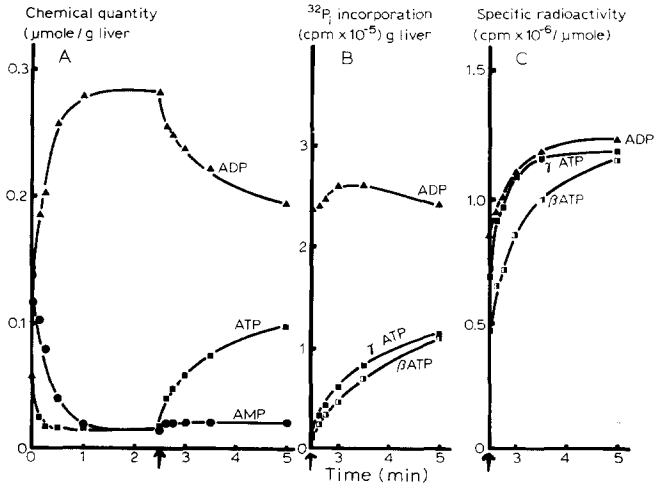


Fig. 6. The conversion of ^{32}P-labelled ADP to ATP. Mitochondria from 600 mg rat liver were incubated in 6 ml reaction mixture containing 5 mM glutamate, 0.1 mM $^{32}P_i$ and 0.02 mM 2,4-dinitrophenol. At 2.5 min, 0.3% bovine serum albumin (Fraction No. V) was added together with additional 5 mM glutamate as indicated by arrow and incubation was continued up to 5 min. The chemical quantities of ATP (■), ADP (▲) and AMP (●) are plotted in Panel A, while the periodical changes in ^{32}P contents of γ-phosphate of ATP (■), β-phosphate of ATP (□), and ADP (▲) after the addition of albumin are plotted in Panel B. Their specific radioactivities are shown in Panel C (the same symbols as in Panel B). Each point represents the mean of two observations.

of ATP concurrently with the conversion of ADP to ATP. Under this condition, the specific radioactivity of ATP with respect to β-position attained the level roughly equal to that of the precursor ADP (Fig. 6C). This result indicates that the molecules of ADP generated from AMP serve as the phosphate acceptor for the oxidative phosphorylation as efficiently as those originally present in mitochondria or those that have originated from ATP by virtue of 2,4-dinitrophenol-induced ATPase. In other words, there is no indication that a part of ADP might be in a different compartment, in accord with the conclusion of Heldt and Pfaff[12] based on their experiments on the translocation of exogenous ADP. It can be said, therefore, that the substrate-level phosphorylation plays a significant role in supplying the phosphate acceptor for the respiration-linked phosphorylation in rat liver mitochondria.

DISCUSSION

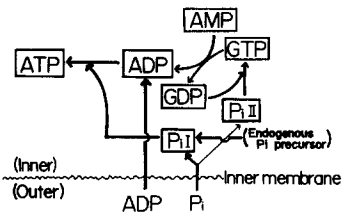
Since the decrease in mitochondrial AMP content observed during incubation was always found to be accompanied by a stoichiometric increase in ATP and/or ADP content, it can be regarded as reflecting the phosphorylation of AMP. The first part of this paper has presented the results which are compatible with the view that this phosphorylation of endogenous AMP is closely related to the substrate-level phosphorylation linked to the oxidation of 2-oxoglutarate. One of the most significant findings in this regard was that the addition of 2,4-dinitro-

phenol promoted the phosphorylation of AMP (Fig. 1). This finding not only shows that the phosphorylation of AMP is distinct in nature from the oxidative phosphorylation but also excludes the possibility that ATP serves as the phosphate donor for the AMP phosphorylation. Therefore, an involvement of adenylate kinase or adenylate kinase-like activity exhibited by a combined function of GTP:AMP phosphotransferase with nucleoside diphosphokinase[13,14] in the phosphorylation of AMP is safely excluded. Instead, the prevention of AMP phosphorylation, caused either by the addition of arsenite (Table I) or by substituting succinate for glutamate as respiratory substrate (Fig. 1D), lends support to the view that 2-oxoglutarate oxidation is concerned in the phosphorylation. Thus, the present results, when coupled with the preceding tracer studies[2] showing that GTP serves as a precursor of ADP in the intramitochondrial phosphorylation reaction which occurs in correlation with the oxidation of glutamate, have afforded convincing evidence that the phosphorylation of endogenous AMP proceeds coupled to the oxidation of 2-oxoglutarate in the inner space of rat-liver mitochondria; GTP generated by succinyl-CoA synthetase being then utilized for the phosphorylation of AMP.

The following findings form the experimental basis for our postulate that the phosphorylation of AMP is supported by the P_i pool which is compartmentalized in such a fashion as to communicate less readily with external P_i than the P_i pool serving as the direct substrate of ADP phosphorylation in the oxidative phosphorylation. Firstly, $^{32}P_i$ was incorporated into ADP more slowly than into ATP even when the phosphorylation of AMP occurred at a rate comparable to the phosphorylation of ADP (Figs 3 and 4). Secondly, [^{32}P]ADP, once formed, lost its ^{32}P more slowly than [^{32}P]ATP when mitochondria were incubated with non-radioacitive P_i (Fig. 5). Thirdly, the addition of 0.1 mM P_i was very effective in raising the ratio of ATP to ADP in mitochondria, while it failed to increase the ratio of ADP to AMP. Since the incubation of mitochondria with 2,4-dinitrophenol brought forth a markedly increased ratio of ADP to AMP (Fig. 1), despite a significant decrease in the size of the intramitochondrial P_i pool (Table III) presumably caused by the uncoupler-induced outflow of P_i[15,16], it appears that the phosphorylation of AMP, apart from the phosphorylation of ADP, is not supported by the major part of intramitochondrial P_i. It is true that not P_i but GTP is a direct phosphate donor for the phosphorylation of AMP. However, the net phosphorylation of AMP should eventually be accompanied by a net supply of P_i, because only a minute amount of GTP (and GDP), less than some per cent of adenine nucleotides, is available in rat liver mitochondria[17]. It can be concluded, therefore, that the phosphorylation of AMP is selectively supported by a minor pool of P_i separated, functionally or spatially, from the major pool which expands or diminishes in response to the inflow or outflow of P_i through P_i transporter, respectively.

A hypothetical relationship between nucleotides and P_i pools inside the inner membrane based on this postulate is simply summarized in Scheme 1, where it is assumed that external P_i enters the P_i Pool I rapidly but its entry into the P_i Pool II occurs only slowly. (The routes of flow of substrates are shown by the arrows connecting the pools, the thickness of arrow representing the relative rate of flow.) P_i in Pool I serves as the substrate of oxidative phosphorylation while P_i in Pool II is utilized for the phosphorylation of GDP by succinyl–CoA synthetase. The

differential P_i requirements for substrate-level phosphorylation and respiration-linked phosphorylation might be explained alternatively in terms of different affinities of these phosphorylation reactions for a single P_i pool. However, the rapid conversion of AMP to ADP observed upon the addition of 2,4-dinitrophenol (Fig 1), which effectively lowers P_i level in mitochondria (Table III), shows that the intramitochondrial concentration of P_i is sufficiently high for maintaining the substrate-level phosphorylation. Hence, the postulation of a compartmentalized P_i pool appears to be a more likely explanation for the present findings.



Scheme 1. A hypothetical compartmentation of intramitochondrial P_i pool and its relation to adenine nucleotides.

Moreover, the postulate of compartments in the endogenous pool of P_i appears to prove useful as well in explaining why the addition of ATP or ADP to the incubation medium results in an almost exclusive $^{32}P_i$ labelling of γ-phosphate of ATP (without significant labelling of ADP) as has been so far observed by many investigators. The simplest explanation for the lack of $^{32}P_i$ labelling of ADP under this condition might be that the addition of ATP or ADP efficiently inhibits the phosphorylation of AMP. However, we found that the phosphorylation of AMP proceeded at the same rate regardless of whether ATP was present or absent in the reaction mixture (to be published). Nevertheless, the incorporation of $^{32}P_i$ into the ADP fraction was strongly suppressed in the ATP-added medium. In other words, the incorporation of $^{32}P_i$ into the ADP fraction was out of proportion to the phosphorylation of AMP in the presence of exogenous ATP. This peculiar phenomenon may be accounted for as follows in terms of the difference in specific radioactivity between two P_i pools which support the phosphorylation of ADP and AMP separately.

As is shown in Table III, P_i in the inner space of mitochondria did not show a decline during incubation without external P_i, suggesting that P_i utilized for phosphorylation is compensated by a continuous supply from the endogenous sources, probably mitochondrial organic phosphate compounds such as phospholipids and phosphoproteins. It is likely that P_i from such endogenous P_i precursors enters into the P_i Pool I, as illustrated in Scheme 1. It is conceivable, therefore, that the specific radioactivity of P_i in Pool I is higher initially, but later it becomes lower than that of P_i in Pool II during incubation without enormous amount of P_i. This situation may give a good explanation for the finding that the specific radioactivity of ADP as a whole eventually attained to a level comparable to the specific activity of γ-phosphate of ATP (Fig. 3C), despite the fact that a considerable amount of ADP was present when the phosphorylation of AMP started, while essentially no ATP was present at the onset of the phosphorylation of ADP. In con-

trast, the specific radioactivity of P_i in Pool I appears to be kept much higher than that of P_i in Pool II during the entire course of incubation with external ATP or ADP (*plus* P_i), because the addition of ATP or ADP (*plus* P_i) promotes the turnover of P_i Pool I (inflow of P_i from external space and its outflow to the phosphorylation site) by virtue of the acceleration of oxidative phosphorylation, thereby making the inflow of non-radioactive P_i from endogenous sources negligible. Thus, under this condition, the phosphorylation of AMP is associated with only a slight incorporation of $^{32}P_i$ into ADP because of the relatively lower specific radioactivity of the precursor P_i which is compartmentalized in Pool II, being prevented from readily communicating with the externally added P_i.

Further studies are now in progress on the interaction between the substrate-level and respiration-linked phosphorylations in rat liver mitochondria.

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