Biochimica et Biophysica Acta, 292 (1973) 310–324

BBA 46470

THE INCORPORATION OF $^{32}P_i$ INTO INTRAMITOCHONDRIAL ADP FRACTION DEPENDENT ON THE SUBSTRATE-LEVEL PHOSPHORYLATION

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(Received July 27th, 1972)

SUMMARY

1. A significant amount of $^{32}P_i$ is incorporated into ADP fraction if mitochondrial phosphorylation is allowed to proceed solely dependent on the endogenous adenine nucleotides even in the absence of uncouplers or inhibitors of oxidative phosphorylation. This formation of [^{32}P]ADP is accompanied by a significant labelling of the GTP fraction as well as by a decrease in mitochondrial AMP.

2. A good correlation, highly significant on a statistical basis, is obtained between the incorporation of $^{32}P_i$ into ADP on the one hand and the oxidation of [1-^{14}C]glutamate to $^{14}CO_2$ on the other, under a wide variety of conditions of respiration, suggesting that the substrate-level phosphorylation linked to the oxidation of 2-oxoglutarate leads to the phosphorylation of AMP in rat liver mitochondria.

3. Since intramitochondrial GTP is not directly labelled by the [^{32}P]ATP added, it is concluded that neither nucleoside diphosphokinase (ATP: nucleoside diphosphate phosphotransferase, EC 2.7.4.6) nor adenylate kinase (ATP: AMP phosphotransferase, EC 2.7.4.3) is functioning in such an EDTA-containing medium as employed in the present study because of lack of the enzymes inside the inner membrane. This not only indicates that ATP never serves as a phosphate donor for the observed phosphorylation of AMP, but also, along with several other lines of evidence, lends strong support to the view that [^{32}P]GTP generated as a result of the substrate-level phosphorylation is a direct precursor of [^{32}P]ADP through the mediation of GTP: AMP phosphotransferase, which has been verified to be located inside the inner membrane by the significant labelling of GTP by [^{32}P]ADP.

INTRODUCTION

It was first shown by Heldt and Schwalbach[1] that the anaerobic dismutation of 2-oxoglutarate in the presence of oligomycin and $^{32}P_i$ led mainly to the labelling of the ADP rather than the ATP fraction of rat-liver mitochondria. They gave convincing evidence that the formation of [^{32}P]ADP under this condition is strictly dependent on the substrate-level phosphorylation linked to the oxidation of 2-oxoglutarate; GTP generated by succinyl-CoA synthetase (succinyl-CoA ligase

(GDP), EC 6.2.1.4) served as a phosphate donor for the phosphorylation of AMP *via* GTP-AMP phosphotransferase according to the following equations.

$$2\text{-Oxoglutarate} + NAD^+ + CoA \rightleftharpoons \text{succinyl-CoA} + NADH + H^+ + CO_2 \quad (1)$$

$$\text{Succinyl-CoA} + GDP + P_i \rightleftharpoons \text{succinate} + CoA + GTP \quad (2)$$

$$GTP + AMP \rightleftharpoons ADP + GDP \quad (3)$$

Owing to the strict specificity of adenine nucleotide translocase, the endogenous AMP cannot serve as a direct substrate of adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) which is known to exist exclusively outside the adenine nucleotide barrier on mitochondrial inner membrane (see ref. 2 for review). Hence, it is very likely that the 2-oxoglutarate-linked phosphorylation of AMP, observed by Heldt and Schwalbach to function in the matrix space, plays an indispensable role in making the endogenous AMP available as a phosphate acceptor in oxidative phosphorylation in mitochondria. However, in intact mitochondria, the predominant occurrence of the respiration-linked phosphorylation has complicated the design of experiments to evaluate the physiological role of the substrate-level phosphorylation in mitochondrial transphosphorylation reactions. It would appear to have been a general consensus of opinion that the suppression of oxidative phosphorylation by means of an "artificial" or "unphysiological" agent such as oligomycin or uncoupler is a prerequisite for detection of the substrate-level phosphorylation at a significant rate in intact mitochondria.

The present paper, however, shows that, if phosphorylation is allowed to proceed solely dependent on the endogenous nucleotides in a diluted suspension of rat liver mitochondria, $^{32}P_i$ is significantly incorporated into GTP and ADP fractions even in the absence of uncouplers or inhibitors of oxidative phosphorylation. The connection of this $^{32}P_i$ incorporation into ADP with the 2-oxoglutarate-linked substrate-level phosphorylation is the subject of the present study.

MATERIALS AND METHODS

Rat liver mitochondria were isolated by the method of Johnson and Lardy[3] in 0.33 M sucrose–0.1 mM EDTA solution. The standard reaction mixture contained 146 mM KCl, 20 mM Tris–HCl buffer (pH 7.4), 1 mM EDTA, 55 mM sucrose (derived from mitochondrial suspension) and mitochondria from 25 mg rat liver in a final volume of 1.2 ml. Analysis of protein by means of the Folin–Lowry[4] method with use of bovine serum albumin (Fraction No V) as standard revealed that mitochondria from 20 to 25 mg rat liver contained 0.6 mg protein. When the estimation of chemical quantities of adenine nucleotides was undertaken, the reaction mixture was scaled to 5 times the above-mentioned ordinary quantity; mitochondria from 600 mg liver were incubated in 6.0 ml, owing to the restricted sensitivity of the determination method. The reaction was started by the addition of mitochondrial suspension and stopped by $HClO_4$ after continuous shaking at 25 °C for 150 s unless otherwise specified.

Analysis of labelled nucleotides

The supernatant of the deproteinized reaction mixture was added to 20 mg

charcoal and kept for 15 min at 0 °C with occasional shaking. ATP, ADP, AMP and GTP, 1 μmole each, were also added as carriers when incubation was carried out on a smaller scale (0.6 mg protein in 1.2 ml). After washing the charcoal twice with distilled water, the nucleotides were eluted by incubating the charcoal in 4 ml of 60% ethanol containing 0.8% NH_4OH for 30 min at 0 °C. 3 ml of the eluate were then evaporated to dryness under reduced pressure at 25 °C, the residue being dissolved in a small volume of distilled water.

A part of this solution was applied in a 1-cm zone to the polyethyleneimine-cellulose plate (precoated plastic sheet of Cellulose MN300, polyethyleneimine, Macherey Nagel Co., Düren, West Germany) and one-dimensional ascending thin-layer chromatography was carried out at 3 °C by stepwise elution with 0.5 M $(NH_4)_2SO_4$ up to 5 cm above the origin followed by 0.7 M $(NH_4)_2SO_4$ up to 17 cm according to Randerath and Randerath[5]. Spots of nucleotides located under ultraviolet lamp showed R_F values of 0.09–0.12, 0.19–0.23, 0.30–0.35 and 0.45–0.50 for GTP, ATP, ADP and AMP, respectively. After drying the plate in a stream of cool air, each area was cut out, extracted with 0.5 ml of 0.1 M HCl and analyzed for its radioactivity.

Distribution of radioactivity getween P_β and $P\gamma$ of ATP was measured based on the conversion of [^{32}P]ATP to glucose 6-[^{32}P]phosphate by hexokinase as follows. The area corresponding to ATP on the polyethyleneimine–cellulose plate was scraped off and extracted with 2 ml of 0.1 M HCl for 30 s at 0 °C. 0.5 ml of the aliquot was used for the estimation of total γ- and β-phosphate radioactivity of ATP, while 1 ml was transferred to the tubes containing 0.1 ml of 0.6 M Tris, 10 μmoles of glucose, 10 μmoles of $MgCl_2$ and 2 units of yeast hexokinase, the pH of the resultant solution being 7.6 to 7.8. Following incubation at 30 °C for 15 min, 10 mg of charcoal was added together with $HClO_4$ to make a final concentration of 0.1 M and the supernatant was counted as a measure of γ-^{32}P of ATP. The charcoal was washed and eluted as described above to measure β^{32}-P of ATP after being evaporated to dryness *in vacuo*.

Determination of $^{14}CO_2$ liberation and mitochondrial ^{14}C content

In order to estimate the production of radioactive CO_2 from [1-^{14}C]glutamate, the mitochondrial suspension with appropriate additions was incubated in the rubber-stoppered vial equipped with suspending plastic cup. The reaction was terminated by injecting 0.25 ml of $HClO_4$ through the rubber stopper along the wall of the vial to make a final concentration of 0.5 M. Following further addition of hyamine onto a roll of paper in the center cup, the vial was stored at room temperature for 1 to 2 h. Radioactivity trapped by hyamine was then measured. In a parallel experiment conducted under the same condition except for use of open vials instead of the rubber-stoppered ones for incubation, the penetration of [^{14}C]glutamate into mitochondria was measured after separation of mitochondria from the incubation medium by means of the rapid filtration technique on the Millipore filter. After being washed twice with 3 ml of ice-cold sucrose–Tris–KCl–EDTA solution (a mixture of 0.33 M sucrose–0.1 mM EDTA and 0.175 M KCl–0.02 M Tris–HCl, 4:1), the Millipore filter was extracted in 1 ml of 0.1 M $HClO_4$ to be analyzed for its ^{14}C content.

Radioactivity was measured by liquid scintillation counter in toluene-type scintillation fluid (4 g PPO and 0.15 g POPOP in 1 l of toluene) for non-aqueous sam-

ples, or in the 2:1 mixture of the toluene-type scintillator with Triton X-100 for aqueous samples.

Determination of chemical quantities of ATP, ADP and AMP

Following incubation, the nucleotides contained in the $HClO_4$-deproteinized supernatant were condensed by means of the charcoal treatment as described above. The mixture of tracer amounts of ^{3}H-labelled ATP, ADP and AMP was added to all incubation flasks immediately following deproteinization to assess the recovery of adenine nucleotides throughout the condensation procedure. ATP, ADP and AMP were then analyzed enzymatically based on the reaction with hexokinase, pyruvate kinase and myokinase plus pyruvate kinase, respectively, according to Bergmeyer[6], changes in absorbance at 340 nm due to reduction of NADP or oxidation of NADH by the coupled reactions being followed in Gilford spectrophotometer Model 2400.

Preparation of [γ-^{32}P]ATP and [^{32}P]ADP

[γ-^{32}P]ATP was prepared by means of mitochondrial P_i–ATP exchange reaction as follows. Mitochondria from 50 mg rat liver were incubated in the EDTA-containing KCl–Tris solution with 0.01 mM $^{32}P_i$ and 0.1 mM ATP in the absence of Mg^{2+} in a final volume of 2.0 ml. Reaction was terminated by the addition of $HClO_4$ and the nucleotide fraction was prepared by treatment with charcoal as described above. Chromatographic separation on polyethylenimene–cellulose plate revealed that the total ^{32}P of this preparation consisted of 94.8% γ-phosphate of ATP, 3.2% β-phosphate of ATP, 1.8% ADP and 0.1% GTP. Accordingly, this preparation was used as [γ^{32}-P]ATP without further purification, an appropriate correction based on these contaminants being made for assessment of ^{32}P incorporation.

In order to prepare [^{32}P]ADP, AMP was phosphorylated by [^{32}P]ATP thus prepared by means of myokinase. Following centrifuging off the myokinase protein denatured with $HClO_4$, ATP which survived myokinase reaction was converted to ADP by yeast hexokinase in the KOH-neutralized supernatant in the presence of 5 mM glucose. The preparation of [^{32}P]ADP thus prepared, though contaminated with non-radioactive AMP, proved to be useful for the present study, because the metabolic pattern within mitochondria was not affected by exogenous AMP below 0.1 mM due to a restricted permeability of mitochondrial membrane to the nucleoside monophosphate.

Chemicals

Adenine and guanine nucleotides, enzymes and coenzymes used as assay reagents were obtained from Sigma. ^{3}H-labelled adenine nucleotides were products of New England Nuclear. Atractyloside was a kind gift from Dr A. Bruni, University of Padova, Italy.

RESULTS

The pattern of phosphorylation supported by the endogenous nucleotides

In contrast to the almost exclusive labelling of the ATP fraction observed during the incubation of mitochondria in the ATP- or ADP-added medium (*e.g.* see Materials and Methods), $^{32}P_i$ was found to be incorporated significantly into the ADP

fraction when the phosphorylation reaction was allowed to proceed dependent only on the endogenous adenine nucleotides by omitting ATP and ADP from the incubation medium. Fig. 1 shows a typical time course of such an endogenous nucleotide-dependent phosphorylation reaction occurring in the reaction mixture fortified with glutamate as a substrate and $K_3Fe(CN)_6$ as an electron acceptor. The intramitochondrial pool of P_i was kept fairly constant throughout the entire incubation time, despite the very low concentration of P_i in the reaction mixture (data not shown but see ref. 7).

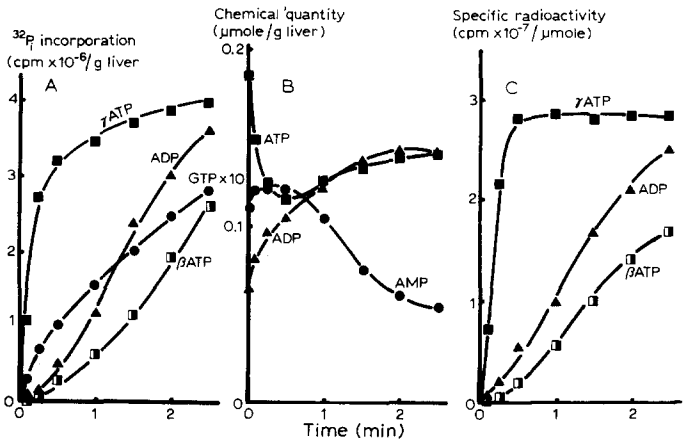

Fig. 1. Periodical changes of ATP, ADP and AMP and their ^{32}P contents during incubation of liver mitochondria with $^{32}P_i$. Mitochondria isolated from 600 mg rat liver were incubated in the standard incubation medium consisting of 0.145 M KCl, 20 mM Tris–HCl (pH 7.4), 1 mM EDTA and 25 mM sucrose in a total volume of 6 ml at 25 °C for a time indicated on abscissa. Glutamate (10 mM), $K_3Fe(CN)_6$ (10 mM) and $^{32}P_i$ were also added. Panel A: the incorporation of $^{32}P_i$ into γ-phosphate of ATP (■), β-phosphate of ATP (◨), ADP (▲) and GTP (●). [^{32}P]GTP was plotted in a scale 10-fold smaller than ^{32}P-labelled adenine nucleotides. Panel B: chemical quantities of ATP (■), ADP (▲) and AMP (●). Panel C: specific radioactivities of γ-phosphate of ATP (■), β-phosphate of ATP (◨) and ADP (▲).

It can be said, therefore, that the phosphorylation dealt with in the present paper represents the phosphorylation of the endogenous nucleotides by the endogenous P_i pool. It is seen in Fig. 1A that, in contrast to a very rapid labelling of the ATP fraction, the incorporation of $^{32}P_i$ into ADP occurred very slowly initially but progressively at higher rates afterwards. The GTP fraction was also labelled under this condition as revealed by a small but sharp peak of ^{32}P in an area corresponding to GTP on the thin-layer plate after fractionation of guanine and adenine nucleotides. (This is a characteristic feature of the phosphorylation observed in the absence of external ATP or ADP, because no significant peak of ^{32}P was detected in the GTP area upon scanning the thin-layer plate on which the products of either phosphorylation of externally added ADP or of the P_i–externally added ATP exchange reaction were fractionated.) The labelling pattern of GTP is recorded in Fig. 1A to a 10-fold larger scale than those of adenine nucleotides.

Fig. 1B shows the periodical change of the chemical quantities of mitochondrial adenine nucleotides. Abrupt breakdown of ATP was observed at the initial stage of incubation causing a simultaneous increase in ADP. During this period (up to 30 s)

the level of AMP was maintained substantially constant, and afterwards, it decreased steadily resulting in a gradual increase of ATP as well as ADP. This pattern of AMP disappearance with an initial lag phase is in good agreement with the pattern of ^{32}P labelling of the ADP fraction (Fig. 1A), suggesting that AMP was actually phosphorylated by the endogenous P_i pool. Since adenylate kinase is not functioning in the EDTA-containing reaction mixture[8], it is probable that AMP was phosphorylated by GTP *via* GTP:AMP phosphotransferase. In accord with the results that not only ADP but also ATP increased as AMP was phosphorylated, β-phosphate of ATP was labelled subsequent to the formation of [^{32}P]ADP (Fig. 1A). Because of the much smaller content of GTP than adenine nucleotides in rat liver mitochondria[9], [^{32}P]-GTP was always lower than [^{32}P]ADP or [^{32}P]ATP. The comparison of the labelling of GTP, ADP and β-phosphate of ATP as a function of incubation time, however, does not appear to be at variance with the view that ^{32}P is transferred from GTP to ADP and then to β-phosphate of ATP successively during the phosphorylation reaction dependent on the endogenous nucleotide pools.

While the specific radioactivity of γ-phosphate of ATP increased and levelled off very rapidly, the specific activity of ADP rose steadily but attained its maximal level roughly as high as that of γ-phosphate of ATP after 3–4 min (the data up to 2.5 min is recorded in Fig. 1C). In contrast, the specific radioactivity of β-phosphate of ATP remained at a much lower level than ADP during incubation.

The correlation of the ^{32}P labelling of ADP fraction to the oxidation of glutamate

The incorporation of $^{32}P_i$ into ADP fraction observed in Fig. 1 was likely to reflect the reaction sequence of Eqns 1 to 3, because it was associated with the significant labelling of GTP. For the purpose of examining this possibility, the relation of this phosphorylation to the oxidation of glutamate was next studied. The liberation of $^{14}CO_2$ from [1-^{14}C]glutamate in the presence of sodium fluorocitrate was used as a simple means of assessing the oxidation of glutamate. The experiments shown in Table I were undertaken first to study the basal requirement for the measured $^{14}CO_2$ liberation to reflect the oxidation of glutamate in mitochondria by means of simultaneously measuring the uptake of [^{14}C]glutamate by mitochondria in a parallel experiment. When mitochondria were incubated with carrier-free [1-^{14}C]glutamate, most (approx. 90%) of the radioactivity taken up by mitochondria was recovered as liberated CO_2 (Group 1 in Table I), suggesting that the inflow of glutamate into mitochondria was limiting the rate of the overall reaction leading to $^{14}CO_2$ production under this condition. Consequently, not only rotenone, an inhibitor of respiration, but also 2,4-dinitrophenol, which must stimulate glutamate oxidation by facilitating the transport of electron, caused an inhibition of CO_2 production as a result of the uncoupler-induced inhibition of the penetration of glutamate into mitochondria[10]. On the other hand, increasing the concentration of glutamate in the medium up to 1 mM by adding carrier glutamate (Group 2) gave rise to an increased transport of glutamate through the mitochondrial membrane, thus rendering the transport step not limiting for the overall process, as evidenced by an accumulation of non-volatile radioactivity in mitochondria relative to $^{14}CO_2$ liberation. In this case, a marked increase in the production of $^{14}CO_2$ was elicited by 2,4-dinitrophenol, with a simultaneous increase in the ratio of CO_2 production to the total radioactivity taken up by mitochondria, indicating that the site of the stimulatory action of 2,4-dinitrophenol

TABLE I

INFLOW OF [1-^{14}C]GLUTAMATE INTO MITOCHONDRIA AND ITS CONVERSION TO CO_2

Mitochondria were incubated in a rubber-stoppered vial and $^{14}CO_2$ liberated during incubation was estimated as described in text. A parallel incubation was done in an open vial under the same conditions to estimate the uptake of [^{14}C]glutamate by mitochondria by means of the rapid filtration technique through the Millipore filter. Sodium fluorocitrate, 0.01 mM, was added to all vials.

Additions	*^{14}C detected (cpm × 10^{-2})*		*$^{14}CO_2$ as % of [^{14}C]glutamate taken up*
	In CO_2 liberated	*Within mitochondria after CO_2 liberation*	
Group 1, without carrier glutamate			
None	543.7	34.9	94.1
2,4-Dinitrophenol (0.05 mM)	223.0	25.1	90.0
Rotenone (0.2 μM)	272.5	62.3	88.7
Group 2, with 1 mM glutamate as carrier			
None	31.5	43.8	41.8
2,4-Dinitrophenol	61.1	31.3	66.2
Rotenone	20.4	38.3	34.8
Group 3, with 1 mM glutamate and 1 mM arsenite			
None	12.6	62.3	16.8
2,4-Dinitrophenol	11.5	34.3	25.2
Rotenone	9.2	39.3	19.9

was located on the reaction responsible for the conversion of radioactive precursors to $^{14}CO_2$ within mitochondria. A rather direct effect of 2,4-dinitrophenol on the mitochondrial translocation system for substrate anions was observed in Group 3 of Table I, where arsenite was added together with fluorocitrate to make the uptake of radioactive glutamate (added at 1 mM) largely dependent on the transport process by arresting further metabolism within mitochondria. 2,4-Dinitrophenol caused a decreased accumulation of radioactivity in mitochondria under this condition, confirming the previous findings that uncouplers are inhibitory to the translocation of substrate anions such as succinate[10,11,12], citrate[10,12,14], malate[10,12–14], 2-oxoglutarate[10,12], pyruvate[12] and glutamate[10] through the membrane of mitochondria.

Thus, Table I shows that the rate of production of $^{14}CO_2$ in the presence of of 1 mM [1-^{14}C]glutamate and fluorocitrate can be used as an index of the ability of mitochondria to oxidize glutamate to succinyl-CoA largely independent of the transport step. Fig. 1 summarizes the results of a lot of experiments in which the change in the rate of oxidation of glutamate, estimated by the thus established means, was correlated with the change in the incorporation of $^{32}P_i$ into mitochondrial ADP fraction. The activation of glutamate oxidation was caused by the addition of 2,4-dinitrophenol, valinomycin or electron acceptors such as menadione, $K_3Fe(CN)_6$ and methylene blue, while its inhibition was brought about either by adding arsenite

or the inhibitor of respiration such as rotenone and Amytal or by means of depleting mitochondrial K^+, essentially required for the oxidation of respiratory substrates[15–17], by adding 2,4-dinitrophenol to mitochondria suspended in the valinomycin-added and K^+-free medium[16]. The addition of 2,4-dinitrophenol to the mitochondrial suspension at a lower pH (pH 6.0) also caused the inhibition of glutamate oxidation presumably because of the extrusion of mitochondrial K^+ (ref. 18). It is seen in Fig. 2,

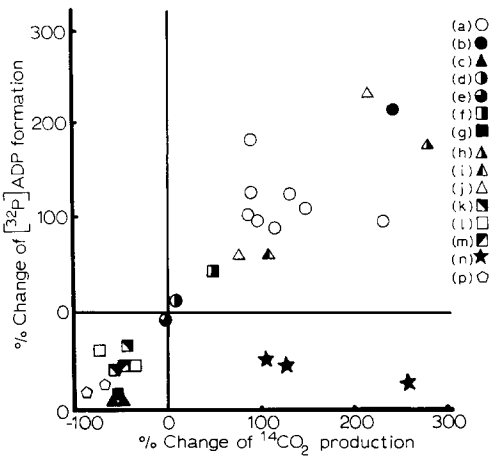

Fig. 2. Effects of various agents on the ^{32}P labelling of mitochondrial ADP fraction as correlated with their effects on the production of $^{14}CO_2$ from [1-^{14}C]glutamate. Each point in the figure represents the percentage effect of the following additions on the incorporation of $^{32}P_i$ into ADP (ordinate) and on the liberation of $^{14}CO_2$ from [1-^{14}C]glutamate (abscissa); (1) 0.05 mM 2,4-dinitrophenol in the standard reaction mixture (a), in mitochondria from 500 mg liver (b), in the medium buffered with imidazole at pH 6.0 (c), in the presence of rotenone (d) or 1 mM phenazine methosulfate (e) and with 1 μg/ml valinomycin in the standard (f) or K^+-free (g) medium. (2) 0.05 mM menadione (h), 10 mM $K_3Fe(CN)_6$ (i) and 2 μM methylene blue (j). (3) 0.2 μM rotenone (k), 2 mM KCN (l) and 0.1 mM Amytal (m). (4) 1 mM sodium arsenate (n). (5) 1 mM sodium arsenite (p).

that the increase or decrease in the rate of glutamate oxidation thus induced was associated with the simultaneous changes of the incorporation of $^{32}P_i$ into ADP to the same direction, respectively, with a statistically significant correlation between these two parameters ($r=0.976$, $P<0.001$). An exceptional case was encountered when arsenate was added; it markedly inhibited the ^{32}P labelling of ADP despite the activated glutamate oxidation. (The data obtained with arsenate was omitted from the calculation of the correlation coefficient.). This is not surprising in view of the fact that arsenate uncouples phosphorylation reactions from not only respiration but also substrate-level oxidative reactions.

It can be concluded, therefore, that the incorporation of $^{32}P_i$ into ADP is closely connected to the oxidation of glutamate. This phosphorylation is very likely to be coupled to the oxidation of 2-oxoglutarate according to Eqns 1 to 3.

Phosphorylation of mitochondrial GDP by exogenous ADP or ATP

The foregoing results have strongly suggested that in rat liver mitochondria, AMP is phosphorylated coupled to the oxidation of 2-oxoglutarate *via* GTP:AMP

phosphotransferase. This is in accord with the report by Heldt and Schwalbach[1], who clearly showed that AMP was phosphorylated prior to ADP when the oxidative phosphorylation was blocked. Since γ-phosphate of ATP was labelled with $^{32}P_i$ prior to ADP under our conditions (Fig. 1, in contrast to the anaerobic experiments carried out by Heldt and Schwalbach[1]), another route of phosphorylation of AMP is still possible; *i.e* the transfer of γ-phosphate of ATP to AMP by way of the following adenylate kinase-like transphosphorylation reaction which has been postulated by Duee and Vignais[19] and Pfaff *et al.*[20] to be in operation in the inner space of rat-liver mitochondria.

$$\text{ATP} + \text{GDP} \rightleftharpoons \text{GTP} + \text{ADP}$$

$$\text{GTP} + \text{AMP} \rightleftharpoons \text{GDP} + \text{ADP}$$

$$\text{Sum: ATP} + \text{AMP} \rightleftharpoons 2\,\text{ADP}$$

In order to examine a possible occurrence of this reaction sequence, we studied the labelling of mitochondrial GTP fraction during incubation with [γ-^{32}P]ATP or [^{32}P]ADP. [γ-^{32}P]ATP and [^{32}P]ADP with high specific radioactivities were used in this experiment, since the mitochondrial pool of GTP is known to be much smaller than the pool of adenine nucleotides[9]. KCN and oligomycin were also added to inhibit the oxidative phosphorylation as well as ATPase. Non-radioactive P_i was also included in the reaction mixture to lower the specific radioactivity of the mitochondrial P_i pool, which might be labelled during incubation as a result of breakdown of [^{32}P]-ATP or [^{32}P]ADP and thereby to minimize the reincorporation of $^{32}P_i$ into GTP

TABLE II

INCORPORATION OF ^{32}P FROM γ-PHOSPHATE OF ATP AND ADP INTO MITOCHONDRIAL GTP

Rat liver mitochondria were incubated in the presence of 0.05 mM [γ-^{32}P]ATP or [^{32}P]ADP with various specific radioactivities in the medium fortified with 1 mM KCN and 10 μM oligomycin. Non-radioactive P_i, 0.1 mM, was also added to all incubation tubes to dilute $^{32}P_i$ which might be liberated during incubation.

Precursor of ^{32}P	*cpm × 10^{-5}/μmole*	*Increase in ^{32}P content of GTP during incubation (cpm)*
ATP	24	248
	48	145
	120	-220
	240	250
ADP	16	395
	32	458
	80	769
	160	1178

dependent on the substrate-level phosphorylation. To make a correction for an insignificant labelling which might be caused by using enormous amounts of radioactivity, multiple measurements were made of the labelling of GTP fraction in the presence of the labelled precursors with varied specific radioactivities; the results being presented in Table II. The small amount of ^{32}P detected in GTP fraction after incubation with [^{32}P]ATP did not increase as the specific radioactivity of [^{32}P]ATP was raised. It can be concluded, therefore, that this label is not significant but only reflects the "background" on the thin-layer plate used for separation of nucleotides. Since the externally added ATP rapidly exchanges with internal adenine nucleotides[21–23] (we actually found that a large part of [^{3}H]ATP added under the same condtion as in Table II was located inside the inner membrane after incubation, data not shown), the possibility can be safely ruled out that our failure to detect ^{32}P in the GTP fraction reflects the event occurring in the translocation process of adenine nucleotide. Instead, these data can be accepted as evidence for a lack of nucleoside diphosphokinase in the inner space. In contrast, the incorporation of ^{32}P from [^{32}P]ADP to GTP showed a graded increase corresponding to an increase of the specific radioactivity of [^{32}P]ADP, indicating that the enzyme reaction catalyzing the transfer of P from ADP to GDP, GTP-AMP phosphotransferase, is operating in the inner space of rat liver mitochondria.

It should be pointed out here that the failure of [^{32}P]ATP to label intramitochondrial GTP additionally affords evidence against the existence of adenylate kinase inside the inner membrane of mitochondria; if adenylate kinase were functioning in this compartment, its combination with GTP:AMP phosphotransferase should have catalyzed the incorporation of ^{32}P from γ-phosphate of ATP to GTP as shown by the following equations:

$$\text{ATP} + \text{AMP} \rightleftharpoons 2\,\text{ADP}$$

$$\text{ADP} + \text{GDP} \rightleftharpoons \text{AMP} + \text{GTP}$$

$$\text{Sum: ATP} + \text{GDP} \rightleftharpoons \text{ADP} + \text{GTP}$$

It is concluded, therefore, that neither nucleoside diphosphokinase nor adenylate kinase is functioning inside the inner membrane and hence that ATP cannot serve as a phosphate donor in the observed phosphorylation of AMP under our experimental conditions, in which transphosphorylation reactions in the outer compartment are blocked by EDTA.

It was noticed that a significant amount of $^{32}P_i$ was released during the course of incubation of mitochondria with [^{32}P]ADP. It appeared, therefore, that [^{32}P]GTP once formed then undergoes hydrolysis according to Eqn 2 in the reversed direction. Actually, [^{32}P]GTP generated from [^{32}P]ADP attained its maximum level at 2 min and then declined (Panel A of Fig. 3). This breakdown of [^{32}P]GTP was accelerated by arsenite and the action of arsenite was further potentiated by succinate. The accelerating action of arsenite and succinate was observed concomitantly in the decrease of [^{32}P]ADP and [^{3}H]ADP (Panel B), in the liberation of $^{32}P_i$ and in the formation of [^{3}H]AMP (Panel C). Since it is highly probable that arsenite, by inhibiting the oxidation of 2-oxoglutarate (Eqn 1), prevents the generation of succinyl

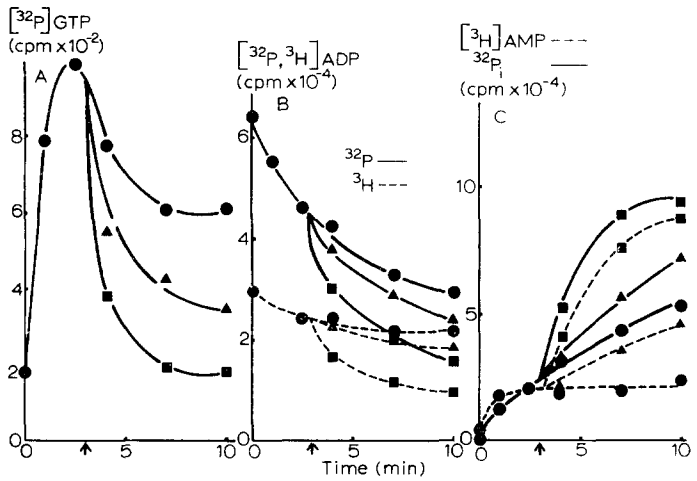

Fig. 3. The transfer of ^{32}P and ^{3}H from doubly labelled ADP. Mitochondria from 24 mg rat liver were incubated at 25 °C in the standard reaction mixture containing 0.145 M KCl, 20 mM Tris–HCl (pH 7.4), 1 mM EDTA and 50 mM sucrose in a total volume of 1.2 ml. 10 nmoles of [^{32}P, ^{3}H]ADP was added at the start of incubation which continued for periods as indicated on abscissa without further addition (●), with 1 mM arsenite (▲) or with arsenite and 10 mM succinate (■) added at a time indicated by arrow. $^{32}P_i$ was precipitated according to the method of Sugino and Miyoshi[39] together with 1 μmole of carrier P_i. The precipitate, after being washed 3 times with the same reagent solution as utilized for preparing precipitate, was dissolved in acetone to be counted in a gas-flow counter. Panel A: ^{32}P content of GTP fraction. Panel B: ^{32}P (———) and ^{3}H (– – – –) content of ADP fraction. Panel C: $^{32}P_i$ (———) and [^{3}H]AMP (– – – –).

CoA and thereby, like succinate, gives rise to a shift of the equilibrium of Eqn 2 from right to left, these results are consistent with the views that ADP undergoes breakdown to AMP and P_i by way of the reactions 3 and 2 in the reversed direction and that the reaction 2 is tightly coupled to the oxidation of 2-oxoglutarate which is sensitive to arsenite.

We are then led to a conclusion that the phosphorylation of AMP, observed to occur in connection with the oxidation of 2-oxoglutarate, resulted from the reaction sequence 1 to 3 in the forward direction with GTP as a compulsory intermediate.

GTP as a precursor of ADP in the $^{32}P_i$ incorporation

The results of the foregoing experiments have been compatible with the view that the incorporation of $^{32}P_i$ into ADP proceeds *via* GTP as a direct precursor according to the reaction sequence 1 to 3 under our conditions, *i.e.* during incubation of mitochondria in ATP- and ADP-free medium. A serious problem in accepting this reaction sequence as a route leading to the labelling of ADP was encountered, however, when it was found that the incorporation of $^{32}P_i$ into ADP fraction was raised but the incorporation into GTP fraction was on the contrary lowered when the oxidation of 2-oxoglutarate was activated by 2,4-dinitrophenol. Such a reciprocal relationship between ADP and GTP fractions in their ^{32}P content, as exemplified at the top of Table III, has been obtained with good reproducibility, seemingly making it implausible to postulate that GTP is a direct precursor of ADP in the substrate-level phosphorylation. Likewise, the enhancement of ^{32}P labelling of ADP caused by

TABIE III

EFFECTS OF 2,4-DINITROPHENOL AND ELECTRON ACCEPTORS ON THE INCORPORATION OF $^{32}P_i$ INTO GTP, ATP AND ADP IN THE PRESENCE OR ABSENCE OF ALBUMIN

Rat liver mitochondria were incubated for 2.5 min with $^{32}P_i$ in the standard reaction mixture in the presence or absence of 0.1% albumin (Fraction No. V). The effects of additions expressed as % of the respective control are presented, positive numerical values representing increases and negative ones decreases.

Additions	*Albumin (0.1%)*	*% effect of additions on ^{32}P content of*		
		GTP	*ATP*	*ADP*
2,4-Dinitrophenol (0.05 mM)	−	−44.5	−67.8	+97.8
	+	+81.5	−45.6	+430.5
Methylene blue (2 μM)	−	−26.7	−32.2	+16.2
	+	+31.2	−13.8	+178.2
Menadione (0.05 mM)	−	−32.4	−47.7	+58.9
	+	+37.9	−19.1	+232.0

electron acceptors such as methylene blue and menadione was accompanied by the lowering of [^{32}P]GTP as shown in Lines 3 and 5 in Table III.

It was found, however, that the addition of 0.1% albumin was very effective in overcoming this contradictory situation, *i.e.* both [^{32}P]GTP and [^{32}P]ADP were increased by 2,4-dinitrophenol or electron acceptors when the incubation medium was supplemented with 0.1% albumin. Fig. 4 illustrates the relationship between the mitochondrial levels of [^{32}P]GTP and [^{32}P]ADP as a function of 2,4-dinitrophenol concentration when mitochondria were incubated with $^{32}P_i$ in the presence (Panel B)

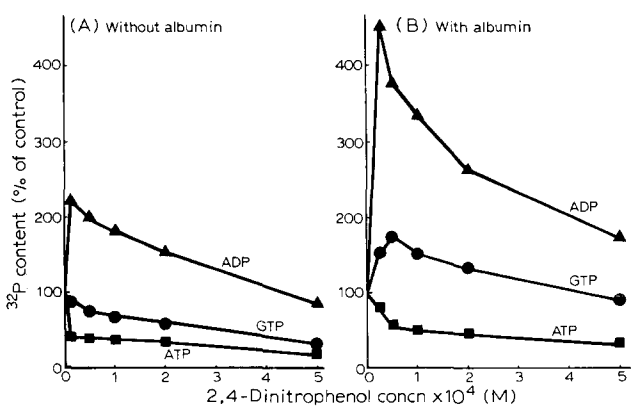

Fig. 4. The incorporation of $^{32}P_i$ into GTP, ATP and ADP as a function of 2,4-dinitrophenol concentration. Rat liver mitochondria were incubated with $^{32}P_i$ in the standard reaction mixture for 2.5 min in the presence of 2,4-dinitrophenol at concentrations indicated on abscissa. The results are recorded as percentage of the control value obtained without 2,4-dinitrophenol. Panel A: without albumin. Panel B: with 0.1% albumin. ■, ATP; ▲, ADP; ●, GTP.

or absence (Panel A) of albumin. The level of [^{32}P]ATP was also included in Fig. 4 which shows that the incorporation of $^{32}P_i$ into ATP was inhibited at all concentrations of 2,4-dinitrophenol, even in the presence of 0.1% albumin. Serum albumin is known to prevent the deleterious action of uncouplers such as 2,4-dinitrophenol[24,25], pentachlorophenol[25,26] and dicoumarol[25,27] on oxidative phosphorylation due to its ability of binding these reagents. The failure of albumin to abolish the inhibitory action of 2,4-dinitrophenol on the [^{32}P]ATP formation, as observed in Fig. 4, may be explainable on the basis of the fact that the concentration of albumin (0.1%) employed here was too low to bind all the 2,4-dinitrophenol molecules. In fact, it was reported that the abolition by 0.3% albumin of the inhibitory action of 2,4-dinitrophenol on the mitochondrial phosphorylation was observed only when the concentration of 2,4-dinitrophenol was below 0.03 mM[24]. It can be seen in Fig. 4 that, in the absence of albumin, [^{32}P]GTP level was lowered by 2,4-dinitrophenol at all concentrations despite the significant elevation of [^{32}P]ADP level. In contrast, in the presence of albumin, [^{32}P]GTP level was raised by 2,4-dinitrophenol in a roughly parallel fashion to the change in [^{32}P]ADP.

Thus, it can be said that mitochondrial GTP behaves in a manner appropriate for a direct precursor of ADP in the presence of albumin, when ADP formation is accelerated by uncouplers or electron acceptors.

A decline of ^{32}P content of GTP observed upon the activation of electron transport (induced by the addition of uncoupler or electron acceptor) in the albumin-free medium might be explained as follows. Under this condition, an active regeneration of NAD caused by feeding electrons to the respiratory chain results in the oxidation of not only 2-oxoglutarate but also fatty acids. Since the oxidation of fatty acids is preceded by the activation of fatty acids to acyl-CoA, GTP would undergo a breakdown *via* GTP-dependent acyl-CoA synthetase[28–30]; the enzyme located inside the inner membrane[31]. The experimental basis for this postulate was found in the facts that the action of 2,4-dinitrophenol or electron acceptors in this respect was mimicked by palmitate (the addition of palmitate caused 62.9% decrease in [^{32}P]GTP with 88.8% increase in [^{32}P]ADP) and that palmitate-bound albumin, unlike fatty acid-free albumin, was without effect in restoring the labelling of GTP (data not shown). If an assumption is made that intramitochondrial GTP accessible to GTP-specific fatty acyl-CoA synthetase is compartmentalized in such a manner as to be prevented from directly communicating with the GTP molecule, which serves as a direct precursor of ADP *via* GTP:AMP phosphotransferase, the incorporation of $^{32}P_i$ into ADP could be promoted even when the label of the mitochondrial pool of GTP as a whole is reduced by virtue of an activation of the endogenous fatty acids. Once the endogenous fatty acids are removed by added albumin, labelling of the whole GTP pool could reflect the activity of the substrate-level phosphorylation in a parallel fashion to the labelling of ADP.

DISCUSSION

The medium employed here for incubation of mitochondria has two characteristics; one is the omission of adenine nucleotides permeable through mitochondrial membrane such as ATP or ADP while the other is the presence of EDTA. We found that both of these characteristics are responsible for the observed phosphorylation

patterns. Since the outflow of the internal adenine nucleotides occurs only in an exchange for the external ATP or ADP through membrane translocase, almost all of the nucleotides labelled in the present study were found to be retained inside the inner membrane. (By means of the rapid filtration technique through the Millipore filter[21,22], our typical experiment showed that 94.5, 73.8, 76.3, 95.4, 89.4, and 84.7% of [^{32}P]GTP, [^{32}P]ATP, [^{3}H]ATP, [^{32}P]ADP, [^{3}H]ADP and [^{3}H]AMP, respectively, were inside the inner membrane after incubation (for 3 min) with $^{32}P_i$ and a tracer amount of [^{3}H]ADP). Likewise, the addition of EDTA was effective in inhibiting the transphosphorylation reaction in the outer space as evidenced by the data in Table II; neither adenylate kinase nor nucleoside diphosphokinase, both of which are known[32–38] to be located in the outer space, functioning under the present condition. Thus, it can be safely concluded that the pattern of phosphorylation observed in the present study strictly concerns the phosphorylation of the endogenous nucleotides inside the inner membrane. This conclusion was confirmed by the fact that the addition of atractyloside, a strong inhibitor of translocase, to the ATP- or ADP-containing medium gave rise to a phosphorylation pattern exactly the same as those in the ATP- or ADP-free medium (data not shown).

The phosphorylation reaction dependent on the endogenous nucleotides prevailing under the present condition is characterized by a significant $^{32}P_i$ labelling of ADP rather than ATP fractions. The experimental results presented here have provided evidence that this phosphorylation reflects the substrate-level phosphorylation linked to the oxidation of 2-oxoglutarate by way of the reaction sequence represented by Eqns 1 to 3. It was also shown that the phosphorylation of AMP actually occurred concurrently with the incorporation of $^{32}P_i$ into ADP. Thus, the present results involve not only a confirmation but also an important extension of the original finding by Heldt and Schwalbach[1], who showed that an anaerobic dismutation of 2-oxoglutarate leads to the labelling of ADP rather than ATP when oxidative phosphorylation is blocked by oligomycin. Since our system does not contain any "unphysiological" reagent which must disturb the energy-dependent reactions in mitochondria, further studies on the phosphorylation reactions in this system will make it very promising to gain an insight into the important relationship between the respiration-linked and the substrate-level phosphorylations. An example of such studies will be presented elsewhere[7].

In contrast to the phosphorylation dependent on the endogenous nucleotides, the phosphorylation observed with 0.1 mM (or more) ATP or ADP externally added is characterized by an essentially exclusive labelling of ATP fraction, as has been observed by many investigators. We feel that the predominant occurrence of respiration-linked phosphorylation evoked by the addition of either the phosphate acceptor (ADP) or the substrate of the terminal exchange reaction (ATP) of oxidative phosphorylation masks the substrate-level phosphorylation. The exact nature of this masking will be discussed in the subsequent paper[7] in terms of compartmentalized P_i pools in rat liver mitochondria.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial support from Eli Lilly and Company for a part of this work. We are indebted to Dr A. Bruni for his kind gift of atractyloside.

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