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Kozo Inaba* Takuzo Oda†

*Okayama University,

†Okayama University,

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Abstract

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PHOSPHORYLATION OF PURINE AND PYRIMIDINE NUCLEOSIDES BY ISOLATED RAT LIVER MITOCHONDRIA

Kozo INABA* and Takuzo ODA

Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama, Japan

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Abstract: Formation of 5′-AMP, 5′-GMP, 5′-CMP and 5′-UMP was confirmed in isolated rat liver mitochondria incubated with α-ketoglutarate, inorganic phosphate, purine nucleoside and pyrimidine nucleoside. Increased incorporation of ^{32}Pi into ATP, GTP and UTP was observed by adding purine- and pyrimidine nucleosides. The phosphorylation of nucleosides was inhibited severely by arsenite and affected slightly by the addition of nuclear or post-mitochondrial fraction.

There are few reports on the physicochemical analysis of RNA synthesized by isolated mitochondria since the rate of RNA synthesis in isolated mitochondria is so low that this system can hardly be used for analysis. It is, therefore, important to examine the incubation mixture system for RNA synthesis of mitochondria. Purine- and pyrimidine nucleoside triphosphates have been used as substrates of RNA synthesis in most cases of isolated mitochondrial incubation. These nucleotides, however, are very poor substrates since the compartmentation of nucleotide transport in mitochondria (1) and the transport of nucleotides into mitochondria are very restrictive. Swelling treatment or prolonged incubation for removing the restriction of nucleotide transport results in strong activation of latent ATPase.

It is well known (2) that ^{32}Pi is incorporated into purine- and pyrimidine nucleoside di- and triphosphates of endogenous nucleotides provided rat liver mitochondria are incubated with α-ketoglutarate and ^{32}Pi, but the α-position of these nucleotides is not phosphorylated.

The present paper reports that ^{32}Pi is incorporated into the α-position of purine- and pyrimidine nucleotides in isolated rat liver mitochondria aerobically incubated with ^{32}Pi in the presence of exogenous α-ketoglutarate and purine- and pyrimidine nucleosides. An increased incorporation of ^{32}Pi into ATP, GTP and UTP is observed by adding the corresponding nucleoside.

* Present address: Department of Physiology, Okayama University Medical School

These incorporations are free from the affect of nuclear or post-mitochondrial contaminants.

MATERIALS AND METHODS

Materials. Rats of the pure inbred Donryu strain were used. ^{32}Pi (carrier free) and ^{3}H-uridine (1-3 Ci/mmole) were obtained from the Japan Atomic Energy Institute and the Radiochemical Center, respectively. Purine- and pyrimidine nucleosides were products of Nakarai Pure Chemicals, Japan. Nucleotides and Dowex 1×2 were purchased from Sigma Chemical Co.

Preparation of mitochondria and incubation mixture. Mitochondria were isolated from rat liver by a slight modification of the method of Hogeboom (3). The purified mitochondria (20-25 mg protein) were suspended in the incubation medium if not stated otherwise. This purified mitochondria contained 170 mM sucrose, 5 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1% bovine serum albumin, 10 mM potassium α-ketoglutarate, 2 mM ^{32}Pi (25 μCi/μmole, pH 7.4) and with or without purine- and pyrimidine nucleosides (4 mM adenosine, 1 mM guanosine, 2 mM cytidine and 2 mM uridine). The final volume of the incubation mixture was 10 ml. Incubation was carried out for 30 min at 25°C under gentle shaking and stopped by placing the incubation flask in an ice bath.

Acid soluble fraction of mitochondria. The mitochondria were collected from the incubation mixture by centrifuging at 7,500×g for 10 min at 0°C. To the mitochondrial pellet 1 N perchloric acid was added at a final concentration of 0.8 N; the mixture was stirred with a glass rod for 20 min at 0°C and centrifuged at 7,000×g for 10 min. The supernatant was saved and the precipitate was washed with a small volume of cold 0.8 N perchloric acid. This supernatant was combined with the original one. The supernatant if not stated otherwise was neutralized with 5 N potassium hydroxide and the precipitate was removed by centrifugation at 3,000×g for 10 min.

The 0.8 N perchloric acid-extract was heated in a boiling water bath for 10 min before neutralization to detect the incorporation of ^{32}Pi into the α-position of purine- and pyrimidine nucleoside di- and triphosphates.

Ion exchange chromatography. The neutralized acid extract was applied to a Dowex 1×2 formate form column (200-400 mesh, 0.8 cm × 25 cm). Elution was performed by passing into the 100 ml mixing chamber containing 50 ml of distilled water, the following in succession: (a) 30 ml of distilled water, (b) 30 ml of 1 N formic acid, (c) 240 ml of 4 N formic acid, (d) 150 ml of 0.2 M ammonium formate in 4 N formic acid, (e) 170 ml of 0.4 M ammonium formate in 4 N formic acid and (f) 100 ml of 1 M ammonium formate in 4 N formic acid. The elution rate was 12 ml/h and 3 ml fractions were collected. Eventhough in most cases positive identification was accomplished by the coincidence of the radioactive peaks from the chromatographic fraction and the authentic marker, the contents of radioactive peaks after lyophilization were chromatographed on paper with appropriate solvents (4-6), and the radioactivity detected by radioautography.

Radioactivity. One ml of the fraction obtained from column chromatography was pipetted to a planchet and dried under an infrared lamp. Measurements were performed with a gas-flow counter (Aloka Inc., Japan). In the case of radioactive materials labeled with ^{3}H, 0.1 ml of each fraction was dissolved in 10 ml of Bray's dioxane scintillator solution (7), and measurements were performed with a Packard Tri-Carb Scintillation Counter (Model 3320) with external standardization.

RESULTS

Rat liver mitochondria were incubated with ^{32}Pi in the presence or absence of exogenous ribonucleosides, as described in the section on Materials and Methods. The acid soluble fraction of mitochondria after incubation was applied to a column of Dowex 1×2. The incorporation of ^{32}Pi into ADP, ATP, GTP and UTP was detected in the endogenous incubation mixture system containing no external nucleosides (Fig. 1).

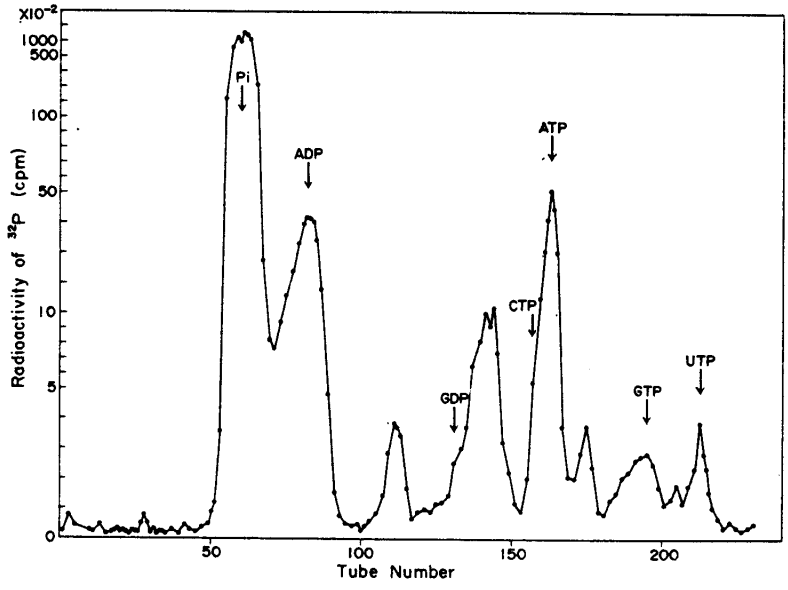

Fig. 1. Column chromatogram of the acid extract of rat liver mitochondria. Mitochondria were incubated aerobically with ^{32}Pi at 25°C for 30 min in the absence of external nucleosides. The acid extract was neutralized and applied to a Dowex 1×2 (formate form) column with added markers, as shown on the figure. Elution was carried out as described in the text.

The incorporation of ^{32}Pi into UTP increased remarkably with the addition of uridine in the incubation mixture (Fig. 2). The addition of adenosine, guanosine, cytidine and uridine resulted in increased incorporation of

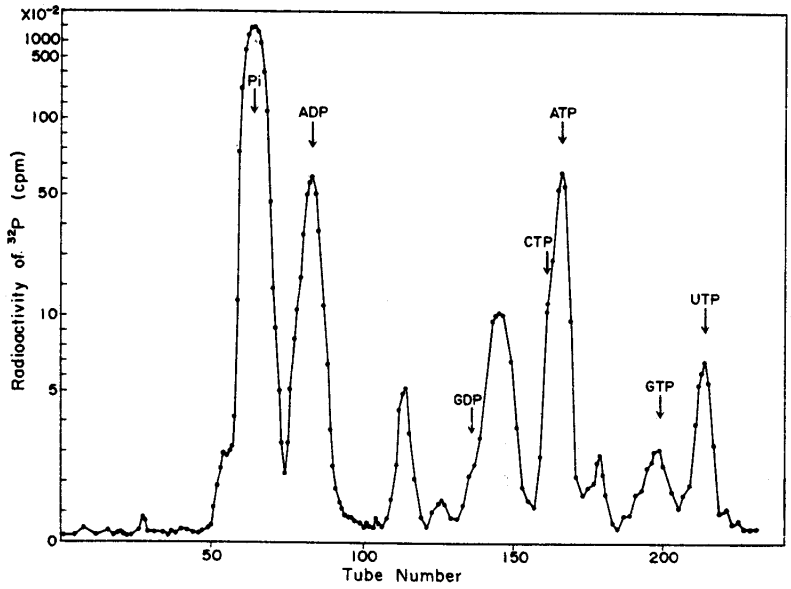

Fig. 2. Column chromatogram of the acid extract of rat liver mitochondria. Mitochondria were incubated aerobically with ^{32}Pi and uridine at 25°C for 30 min. The acid extract was treated as described in the legend of Fig. 1.

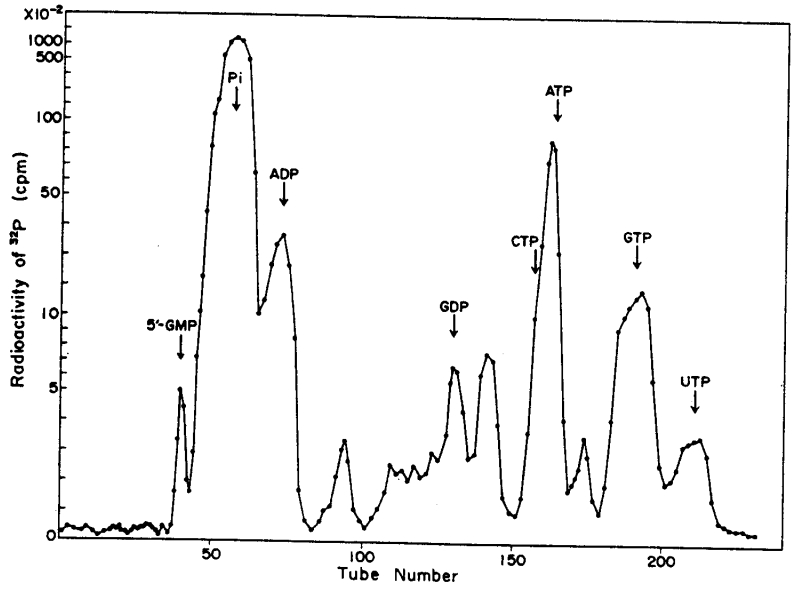

Fig. 3. Column chromatogram of the acid extract of rat liver mitochondria. Mitochondria were incubated aerobically with ^{32}Pi, adenosine, guanosine, cytidine and uridine at 25°C for 30 min. The acid extract was dealt with as described in the legend of Fig. 1.

^{32}Pi into GMP, GDP, ATP and UTP (Fig. 3). The peaks of CDP and CTP overlapped with those of Pi and ATP.

The incorporation of ^{32}Pi into purine and pyrimidine nucleotides was inhibited considerably by the addition of sodium arsenite which is well known as an inhibitor of α-ketoglutarate dehydrogenase (Fig. 4).

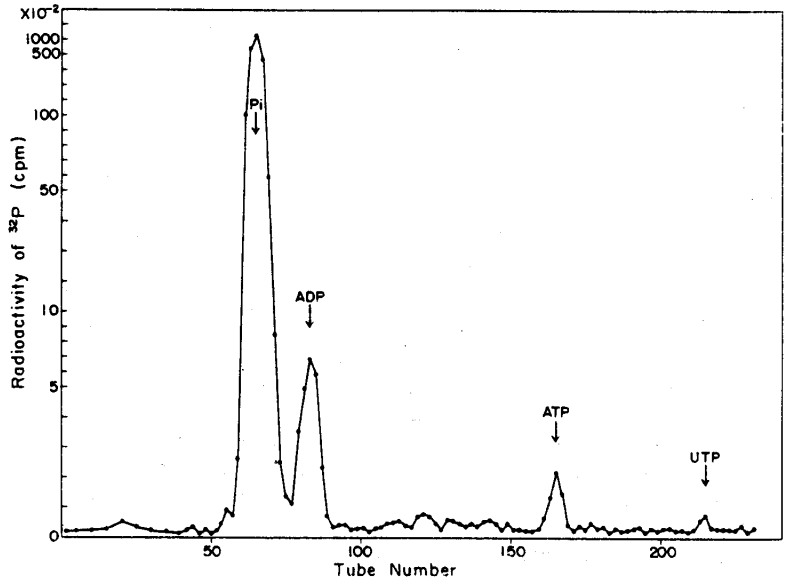

Fig. 4. Column chromatogram of the acid extract of rat liver mitochondria. Mitochondria were incubated aerobically with ^{32}Pi, adenosine, guanosine, cytidine and uridine in the presence of 2 mM sodium arsenite at 25°C for 30 min. The acid extract was dealt with as described in the legend of Fig. 1.

To detect the incorporation of ^{32}Pi into the α-position of purine- and pyrimidine nucleoside di- and triphosphates, after incubation with ^{32}Pi, 0.8 N perchloric acid extract from the mitochondria was heated in a boiling water bath for 10 min. After neutralization and centrifugation, the heat treated-acid extract was applied to a column of Dowex 1 $\times$ 2. The chromatogram is shown in Fig. 5.

CMP, AMP and GMP were detected by the radioactive peaks in the chromatographic fraction and by paper chromatography in the heat treated-acid extract from mitochondria incubated with ^{32}Pi, adenosine, guanosine, cytidine and uridine (closed circles) in contrast to the endogenous incubation mixture system (open circles). In the formation of CMP by mitochondria, cytidine was required and could not be replaced by uridine (triangles).

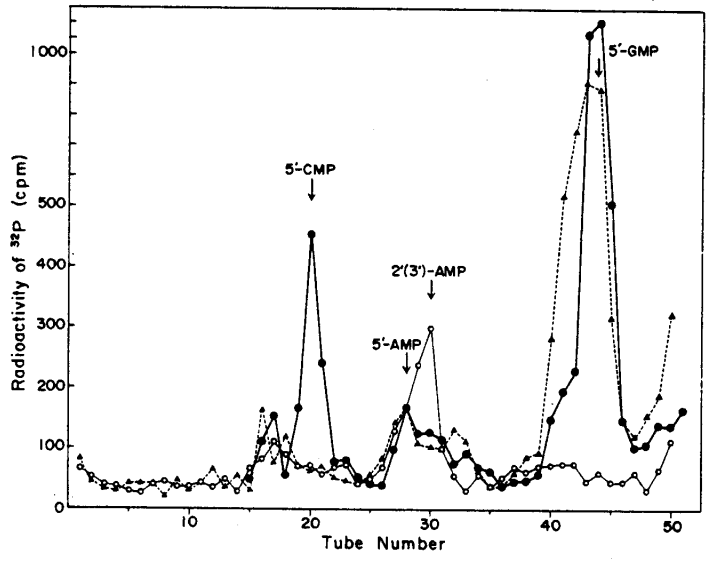

Fig. 5. Column chromatogram of the heat treated-acid extract of rat liver mitochondria. Mitochondria were incubated aerobically with ^{32}Pi in the presence and absence of external nucleosides. The acid extract was heated in a boiling water bath for 10 min and dealt with as described in the legend of Fig. 1. ○——○, without external nucleoside; ▲·····▲, with adenosine, guanosine and uridine; ●——●, with adenosine, guanosine, cytidine and uridine.

Since the peaks of UMP and ^{32}Pi overlapped on the chromatogram, mitochondria were incubated with ^{3}H-uridine and Pi. The chromatogram of the heat treated-acid extract from mitochondria after incubation is shown in Fig. 6. Two peaks were detected besides the first bulk peak of ^{3}H-uridine. The second (or middle) peak has not yet been identified. The last peak was identified as UMP by the elution position and paper chromatography.

It is important to determine whether the contamination of nuclear or post-mitochondrial fraction in the mitochondrial preparation affects the phosphorylation of purine- and pyrimidine nucleosides. Mitochondria were incubated with ^{32}Pi and purine- and pyrimidine nucleosides or with ^{3}H-uridine and Pi with and without the addition of nuclear or post-mitochondrial fraction. It was clear that both the nuclear and post-mitochondrial fractions do not significantly affect the phosphorylation of purine- and pyrimidine nucleosides with mitochondria (Table 1).

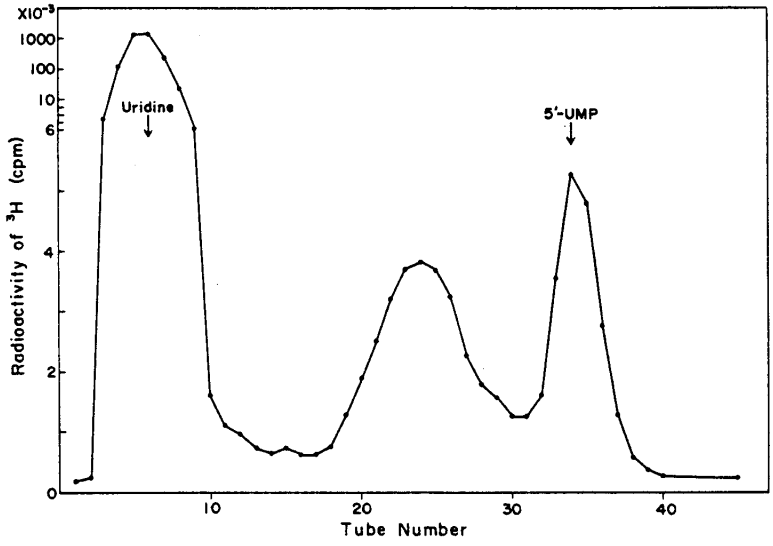

Fig. 6. Column chromatogram of the heat treated-acid extract of rat liver mitochondria. Mitochondria (2 mg protein) were incubated aerobically at 25°C in the incubation medium containing ^{3}H-uridine (20 μCi/mμmole/ml) for 30 min. The acid extract was heated in a boiling water bath for 10 min and applied to a Dowex 1×2 (formate form) column with authentic markers after neutralization. Elution was carried out by passing into the 100 ml mixing chamber containing 50 ml of distilled water the following in succession: (a) 30 ml of distilled water, (b) 30 ml of 1 N formic acid and (c) 70 ml of 0.2 M ammonium formate in 4 N formic acid.

TABLE 1 EFFECT OF NUCLEAR AND POST-MITOCHONDRIAL FRACTIONS ON NUCLEOSIDE PHOSPHORYLATION IN MITOCHONDRIA

Fraction added to incubation mixture	CMP (%)	AMP (%)	GMP (%)	UMP* (%)
None	100	100	100	100
Nuclear fraction**	106	97	95	92
Post-mitochondrial fraction***	106	96	97	90

* Rat liver mitochondria were incubated with ^{3}H-uridine and Pi under the same conditions described in the legend of Fig. 6.

** Nuclear fraction (7 mg protein) obtained from the mitochondrial isolation process.

*** Post-mitochondrial fraction (7 mg protein) obtained from the supernatant after separation of mitochondria.

Rat liver mitochondria were incubated aerobically at 25°C for 30 min with ^{32}Pi, adenosine, guanosine, cytidine, and uridine or ^{3}H-uridine plus Pi, in the presence or absence of a nuclear or post-mitochondrial fraction. The details are described in Fig. 5 and Fig. 6.

DISCUSSION

It has been reported (2) that ^{32}Pi is incorporated into purine- and pyrimidine nucleoside di- and triphosphates, when rat liver mitochondria were incubated with α-ketoglutarate and ^{32}Pi. The present results show that the α-position of the nucleotides was not phosphorylated significantly in the absence of external nucleosides, but by adding purine- and pyrimidine nucleosides, the α-position of the corresponding nucleotides was phosphorylated in coupling with α-ketoglutarate linked phosphorylation. The increased incorporation of ^{32}Pi into ATP, GTP and UTP was also observed by adding nucleosides. The phosphorylation of nucleosides was free from the effects of nuclear or post-mitochondrial contaminants.

There are differences in the results of Tsiftsoglou and Geogratsos (8) and the present study on nucleotide formation in mitochondria. These investigators (8) reported that ^{3}H-cytidine nucleotide was formed when mouse liver mitochondria were incubated for a prolonged period at 37°C with ^{3}H-uridine, ATP, succinate and malate. According to their data, the peaks of labeled nucleoside monophosphate were higher than those of the triphosphates in the acid extract of the mitochondria. The present results indicate that external cytidine is essentially required for the formation of CMP. Furthermore, peaks of labeled nucleoside 5′-monophosphates in the acid extract of mitochondria were very small in contrast to those of nucleoside triphosphates when the acid extract was not heated in a boiling water bath for 10 min. These results may be due to differences in the incubation mixture systems. However, there is the possibility of contaminants in their mitochondrial preparation system (8) since the incubation was carried out for a relatively long time at 37°C and the oxidative phosphorylation would be uncoupled.

What is the most likely pathway for the incorporation of ^{32}Pi into the α-position of purine- and pyrimidine nucleotides in mitochondria? The most probable one seems to be a "salvage pathway." It involves the participations of purine- and pyrimidine nucleoside kinases that are not well defined, coupled with α-ketoglutarate linked-phosphorylation. There is no evidence for the presence of an enzyme in *de novo* synthesis of purine- and pyrimidine nucleoside-5′-phosphates in mitochondria. The incorporation of ^{32}Pi into adenosine-2′(3′)-phosphate observed in the heat treated-acid extract seems to derive from the degradation of NADP formed by the phosphorylation of endogenous NAD.

It is very important to study the physicochemical properties of RNA synthesized in the mitochondrial incubation mixture system. We have already observed that ^{32}Pi is further incorporated into mtRNA in the mitochondrial incubation mixture system. The details will be reported elsewhere.

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