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The Rate of Respiration and Incorporation of P^{32} into Adenosine Triphosphate by Rat Aorta Tissue*

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THE number of publications dealing with the respiration of arterial tissue is still relatively small¹⁾. The primary metabolic activities encompass the fundamental reactions by which the tissue cell derives metabolic energy from the exergonic breakdown of various nutrients. The metabolic energy obtained as adenosine triphosphate (ATP) via a glycolytic cycle and Krebs citric acid cycle is required for the synthetic processes of cellular components. The process of coupled synthesis of ATP from adenosine diphosphate (ADP) and phosphate may thus comprise the main streams of metabolic activity of aorta tissue cells. However, there is a very few study, if any, on the formation of ATP in arterial tissues. The reason for scarcity of investigations on ATP formation must probably be sought in the lesser content of mitochondria and the poorer development of its cristae in aorta tissue compared with those in other tissues, such as heart muscle²⁾³⁾.

Previously, it was demonstrated that the animals fed a magnesium deficient diet showed an enhancement of cholesterol-induced atherosclerosis of aortas⁴⁾⁵⁾ and swelling and a decrease of oxidative phosphorylation of mitochondria of heart muscle³⁾. The studies on arterial wall may contribute to the understanding of atherogenesis. As a preliminary report, the present

study deals with the incorporation rate of orthophosphate- P^{32} into ATP and respirations of aorta tissues of rats under various conditions.

MATERIALS AND METHODS

Animals and diets: Male rats of Douryu strain at different ages were obtained from Tokyo Experimental Animal Laboratory. Rats with various ages were fed a commercial stock ration (Oriental MF) for several days before sacrifice. In the experiments on the effects of feeding a magnesium deficient diet, young albino rats, weighing 45 to 50 gm were fed a basic diet with removing a magnesium from salt mixture as described elsewhere³⁾. Magnesium was added to the control diet in the form of magnesium oxide to supply 48 mg of magnesium per 100 gm of diet. The magnesium deficient diet contained less than 2 mg per cent of magnesium. The rats were housed in the individual cages and fed *ad libitum*. The temperature of animal room was maintained at $20 \pm 1^\circ \text{C}$. The rats were weighed twice weekly and observed daily for 14 to 28 days for signs of magnesium deficiency. At the end of this period, the rats were sacrificed.

Tissue preparations: The rats were sacrificed by decapitation. In the cold room (4°C), the whole aorta was rapidly removed and freed of adherent fat and connective tissue and then cut along its longitudinal axis so as to provide a thin sheet of aorta tissue. The tissues were weighed by torsion balance and stored in a cold Krebs-Ringer-phosphate solution (pH 7.4) until obtaining an enough amount of tissues. This requires usually 10 to 20 minutes

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after sacrifice, particularly more so in young rats experiments, because the pooling of approximately 10 aortas were necessary in one month old rats. In a preliminary experiment, the rate of oxygen uptake of aorta was proportional to the added tissue amount between 200 and 400 mg wet weight of rat aortas under the following conditions. Therefore, in the following experiments approximately 300 mg of wet aorta tissue was used for measuring oxygen uptake and the incorporation rate of P^{32} into ATP. Aortas were blotted on a moist filter paper and transferred to Warburg flasks.

Oxygen uptake and incorporation of P^{32} into ATP: The oxygen uptake was determined by Warburg respirometer at 37°C in an atmosphere of air. The flasks previously contained 2.8 ml of Krebs-Ringer-phosphate buffer solution (pH 7.4), $100\mu\text{M}$ of glucose and orthophosphate- P^{32} (50 to $200\mu\text{C}$ depending on the experiments). Total volume of reaction mixture was 3.0 ml including 0.2 ml of 20% of KOH in a center well. Radioactive orthophosphate (P^{32}) of 99% pure was obtained from the Radiochemical Centre, Amersham, England, through Japan Radioisotope Association, hydrolyzed with 0.1 N HCl for 2 hours at 100°C and neutralized with 0.1 N NaOH. After 8 minutes of thermoequilibration period, manometer readings were performed at 10 minutes intervals and continued for 60 to 150 minutes. At the end of incubation time, arterial tissues were removed quickly from the flasks and washed with an ice cold 0.05 M phosphate buffer (pH 7.4) and then frozen by dry ice-acetone mixture. The frozen tissues were homogenized for three minutes in a glass homogenizer containing 10 volumes of a cold 10% trichloroacetic acid solution and centrifuged. The residues were reextracted with 5 volumes of trichloroacetic acid solution and centrifuged again. The supernatant fractions were combined and treated with 100 mg of activated charcoal (SHIRASAGI) which was treated previously as described elsewhere⁶. The suspended charcoal was separated by centrifugation and washed several times with 5, 2 and 1 per cent trichloroacetic acid solution successively. The charcoal was packed in the column and washed with cold water until the washings showed no significant radioactivity. Then the acid soluble nucleotide fraction was eluted with several portions of 0.1% NH_4OH in 50% ethanol. An aliquot of the eluates was counted by a Geiger Müller counter (Kobe Kogyo Co.) and the remaining eluates were dried under the vacuum and cold conditions. After the acid soluble nucleotide fraction was dried completely, it was dissolved in a minimum amount of distilled water and an aliquot of it was spotted on the filter paper (Toyoroshi No. 51), pretreated with 0.1 N HCl and 0.2 per cent ethylenediaminetetraacetic acid. The chromato-

graphic development was performed at room temperature by the method described by Eggleston and Hems⁷. The paper was dried and the radioactivity was counted by the paper strip scanner (Kobe Kogyo Co., Chromato-beta-analyzer) and then the area corresponding to the standard ATP plus ADP which showed a significant peak of radioactivity in a scanogram, was cut off and counted again by the automatic counting instrument (Kobe Kogyo Co.). Since the separation of ATP and ADP was unsatisfactory in our hand, the final radioactivity of ADP plus ATP spots was recorded as an incorporated radioactivity into ATP fraction and the radioactivity was expressed as c.p.m. per 100 mg of wet tissue. By scanning the radioactivity of the developed paper chromatograms, the contamination of inorganic- P^{32} in the eluted nucleotide fractions was measured and found to be within 10 per cent of the total radioactivity of ADP plus ATP spots.

TABLE I EFFECTS OF STORAGE ON RESPIRATION AND INCORPORATION OF P^{32} INTO ATP OF RAT AORTA

Period of storage (minutes)	QO_2	ATP P^{32} *
0	1.12	2,540
45	0.71	1,450
90	0.64	882
150	0.51	508

Aortas from 2.5 months old male rats were stored in a cold Krebs-Ringer-phosphate solution for time listed in Table. Conditions: approximately 300mg of aorta tissue (wet weight), 2.8ml. of Krebs-Ringer-phosphate solution (pH 7.4) containing glucose ($100\mu\text{M}$) and $50\mu\text{C}$ of orthophosphate- P^{32} and 0.2ml of 20% KOH in center well of Warburg flask were incubated for 60 minutes at 37°C at the presence of air.

* Radioactivity in ATP plus ADP fractions (c.p.m. per 100mg wet tissues).

TABLE II EFFECTS OF METABOLIC INHIBITORS, CYANIDE AND FLUORIDE, ON RESPIRATION AND INCORPORATION OF P^{32} INTO ATP OF RAT AORTA

Experiment No.	Inhibitors (final concentration)	QO_2	ATP P^{32} *
1	none	0.72	9,910
	KCN ($3.5 \times 10^{-3} \text{ M}$)	0.58	4,710
2	none	0.60	12,190
	NaF ($1 \times 10^{-2} \text{ M}$)	0.34	4,930

Conditions were same as in Table I except $100\mu\text{C}$ of P^{32} , 90 minutes of incubation in Experiment 1 and $200\mu\text{C}$ of P^{32} , 60 minutes incubation in Experiment 2. Each value represents the mean of dual experiments.

* See Table I.

TABLE III EFFECTS OF AGE OF RATS ON RESPIRATION AND INCORPORATION OF P^{32} INTO ATP OF RAT AORTA

Experiment No.	P^{32} used per flask (μ c)	One month old rat		Four months old rat		Twelve months old rats	
		Q_{O_2}	$ATP^{32} *$	Q_{O_2}	$ATP^{32} *$	Q_{O_2}	$ATP^{32} *$
1	50	0.64	5,235	0.59	4,775	0.65	1,395
2	100	0.83	9,260	0.69	8,740	0.65	2,455
3	200	1.06	12,085	1.02	7,320	1.12	8,690
4	200	0.87	12,825	—	—	1.12	13,500
5	50	0.83**	4,870**	—	—	0.75***	2,953***
6	200	1.53	33,269	—	—	1.03	19,827

* See Table I. ** Two months old rats. *** 15 months old rats.

TABLE IV EFFECTS OF ACETYLCHOLINE ON RESPIRATION AND INCORPORATION OF P^{32} INTO ATP OF RAT AORTA IN DIFFERENT AGES

Experiment No.	P^{32} used per flask (μ c)	Age of rats (month)	Control		Acetylcholine	
			Q_{O_2}	$ATP^{32} *$	Q_{O_2}	$ATP^{32} *$
1	50	2	0.83	4,870	1.72	9,325
2	200	1.5	0.51	25,000	1.01	46,900
3	50	15	0.75	2,955	0.78	3,090
4	200	12	0.90	27,100	0.90	29,120

Conditions were same as in Table I except acetylcholine ($1 \times 10^{-5} M$) with eserine ($1 \times 10^{-4} M$).

* See Table I.

TABLE V EFFECT OF FEEDING A MAGNESIUM DEFICIENT DIET ON RESPIRATION AND INCORPORATION OF P^{32} INTO ATP OF RAT AORTA

Experiment No.	P^{32} used per flask (μ c)	Control rat aorta		Magnesium deficient rat aorta	
		Q_{O_2}	$ATP^{32} *$	Q_{O_2}	$ATP^{32} *$
1	50	0.91	2,825	0.57	1,178
2	50	0.50	2,360	0.63	2,268
3	100	0.86	5,405	0.49	2,760
4	100	0.60	9,140	0.76	4,890

Conditions were same as in Table I. Periods of feeding a diet were two weeks in Experiment 1 and 2, four weeks in Experiment 3, and 4. Each value represents the mean of dual determinations.

* See Table I.

RESULTS

Effect of storage of tissues in a cold Krebs-Ringer-phosphate solution: In order to measure the oxygen uptake of young rat aorta by Warburg respirometer, approximately 300 mg of tissue had to be used per flask. However, it was anticipated that the metabolic activities of aortas will be destroyed while aortas were preserved in a cold Krebs-Ringer-phosphate solution. The storage effects of rat aorta tissues in a cold Krebs-Ringer-phosphate solution on the measurements of oxygen uptake and the incorporation of P^{32} into ATP were investigated. The decrease of the incorporation rate of P^{32} into ATP was more rapid than that of the

respiration as shown in Table I. It should be emphasized that the distinct decreases in respiration and incorporation of P^{32} into ATP were observed within 45 minutes after sacrifice.

Effects of metabolic inhibitors: Effects of additions of cyanide and/or fluoride on respiration and P^{32} incorporation into ATP were studied as shown in Table II. $3.5 \times 10^{-3} M$ cyanide and $1 \times 10^{-2} M$ fluoride depressed the respiration by 20 and 40 per cent and decreased the incorporation of P^{32} into ATP by 50 to 60 per cent of control values respectively. Differences in the mode of action of these inhibitors upon respiration and incorporation of P^{32} into ATP of young rat aortas were not clear.

Effects of age of rats : In order to determine the effects of age of rats on the metabolic activities of rat aortas, three age groups were chosen, i.e., 1 to 2 months, 4 months, and 12 to 15 months old rats groups. As shown in Table III, Q_{O_2} values of aortas did not differ significantly between each age group, but the rate of incorporation of P^{32} into ATP of aortas of 12 months old rats decreased significantly compared with that of one month old rats except one (Experiment No. 4) out of six experiments. The incorporation rate of P^{32} into ATP of aortas of 4 months old rats was almost same as that of one month old rats in Exp. No. 1 and No. 2 but decreased in Exp. No. 3.

Effects of acetylcholine : In the experiments listed in Table IV, the effects of acetylcholine with eserine were investigated in both young and old rats. When acetylcholine with eserine was added to the incubation medium at the final concentration of $1 \times 10^{-5}M$ (acetylcholine) and $1 \times 10^{-4}M$ (eserine), both the oxygen uptake and the incorporation rate of P^{32} into ATP of aortas from young rats were increased significantly. This enhancement of respiration and P^{32} incorporation rate into ATP by acetylcholine was clearly observed in young rats but not in old rats as shown in Table IV.

Effects of feeding a magnesium deficient diet : In the experiments recorded in Table V, the incorporation rate of P^{32} into ATP was rather decreased in rats fed a magnesium deficient diet for 2 to 4 weeks. Only in one out of four experiments, the incorporation rate of P^{32} into ATP of aortas of magnesium deficient rats was not decreased. The effects of magnesium deficiency on respiration was not observed.

DISCUSSION

KIRK et al.⁸⁾ demonstrated the decrease of respiration of aortas by storage of tissues for two hours to several days after sacrifice. In the present study, it was demonstrated that tissue respiration and particularly the incorporation of P^{32} into ATP of rat aortas were decreased within 45 minutes after sacrifice when the tissues were stored in a cold Krebs-Ringer-phosphate solution at 4°C. The deter-

mination of metabolic activity of aorta tissue should be done within a short time after sacrifice in order to minimize a decrease of activity. However, in the present study, approximately 20 minutes was required after sacrifice until the tissues were placed in Warburg flasks, because an enough amount of tissues had to be pooled in order to measure the respiration accurately by Warburg respirometer. This was more so in the experiments for young rats aortas. The inconsistency of the results of some of the experiments could have been caused by the storage effects. BRIGGS et al.⁹⁾ showed that the oxygen uptake of aorta was depressed by the presence of fluoride, azide and iodoacetate. The present study demonstrated the inhibition of both respiration and P^{32} incorporation into ATP by cyanide and fluoride. There is a disagreement between workers⁸⁻¹¹⁾ concerning the effects of age on respiration of aortas. In the present study, no significant difference in oxygen uptake was noted between the three groups of samples. However, the incorporation rate of P^{32} into ATP of aortas was decreased in old rats compared with that of young rats. This finding may suggest that the metabolic activity of aorta decreases with an increase of age. An increase in a thickness of arterial wall and extracellular materials with an advancement of age, could have caused the decrease of the rate of incorporation of P^{32} into ATP. Determinations of deoxyribonucleic acid (DNA) and ATP in arterial tissues are necessary in better evaluation of this finding and are in progress in our laboratory.

The effects of acetylcholine on the metabolism of arterial tissues were of special interests. Acetylcholine increases a penetration of several substances through cell membrane¹²⁾. In case of old rats, production of ATP is reduced and then probably phosphorylation or synthesis of phospholipids of cell membrane also would be reduced. In fact, it has been observed in our laboratory that total phospholipids of aorta tissues were reduced and the compositions of various phospholipids were changed with advancing of age¹³⁾. The lack of effect of acetylcholine in old rat aortas might be related to these metabolic changes of aorta in old rat.

The feeding of a magnesium deficient diet

with or without cholesterol in a diet, caused an enhancement of experimental atherosclerosis in rabbits⁴⁾⁵⁾, rats¹⁴⁾, puppies¹⁵⁾ and monkeys¹⁶⁾. In the present study, a feeding of magnesium free diet for 2 to 4 weeks developed a significant decrease in the incorporation rate of P^{32} into ATP but not in oxygen uptake of aorta tissues of rats. The contribution of the decrease of ATP formation of aorta tissue in a magnesium deficient animals to the increase of development of experimental atherosclerosis remains to be elucidated. In the different studies, an increase of active phosphorylase of skeletal muscle and a decrease of oxidative phosphorylation of heart muscle were observed in a magnesium deficient rats³⁾¹⁷⁾. It was also found that feeding a magnesium deficient diet did not produce any increase in the absorption of radioactive cholesterol by the blood nor any increase in the radioactivity present in the liver, heart or kidney. However, rabbits fed a magnesium deficient diet showed an increase in the radioactivity detected in the aorta compared with that in the aorta of rabbits fed a control diet¹⁸⁾. From these findings, it seems likely that the enzyme activity producing energy from exergonic catabolism will be insufficient and influx of cholesterol- C^{14} was higher in aortas of magnesium deficient animals.

SUMMARY

Tissue respiration and rate of incorporation of P^{32} into ATP of rat aorta were studied by using Warburg technique in vitro.

1. A decrease of the incorporation rate of P^{32} into ATP was observed within 45 minutes after sacrifice when the aortas were stored in a cold Krebs-Ringer-phosphate solution (pH 7.4).

2. The metabolic inhibitors, cyanide and fluoride, depressed both the respiration and the incorporation of P^{32} into ATP.

3. The incorporation rate of P^{32} into ATP of aortas from 12 to 15 months old rats was lower than that of 1 to 2 months old rats, while no significant difference in Q_{O_2} of aortas

from young and old rats was observed.

4. The enhancement of both the respiration and the incorporation of P^{32} into ATP of rat aorta by acetylcholine was significant in young but not in old rats.

5. The incorporation of P^{32} into ATP was depressed in aortas from rats fed a magnesium deficient diet for 2 to 4 weeks, while the oxygen uptake was not decreased in a significant amount.

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