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Abstract

The energy source required for the amino acid incorporation into mitochondrial proteins has been investigated and comparative study has also been made on the rate of the amino acid incorporation in rat liver and rat hepatoma cell mitochondria. 1. The incorporation of amino acid into the protein in intact mitochondria of rat liver increased by about 40% on the addition of α-ketoglutarate and ADP, but no significant increase in the amino acid incorporation was observed on the addition of succinate and ADP. 2. The incorporation of amino acids into mitochondrial proteins was remarkably inhibited by the addition of respiratory inhibitors (cyanide, DNP at a high concentration). 3. The amino acid incorporation into mitochondrial proteins was scarcely or slightly inhibited by the addition of DNP at the concentration of $1\times$10-4M and insensitive to oligomycin (5 to 10 μg/ml). 4. The amino acid incorporation into the protein in the endogenous substrate system of the mitochondria was considerably inhibited by the addition of arsenite, and this inhibition somewhat recovered on the addition of ADP plus succinate. 5. The rate of the amino acid incorporations between rat liver and hepatoma cell mitochondria was at the same level. 6. Discuions were made on the energy source required for the amino acid incorporation into mitochondrial proteins, on the rate of protein synthesis per mitochondrion isolated from rat liver- and hepatoma cells, and on the poibilities of contamination of bacteria or microsomes and of the adsorption of amino acids onto the mitochondria.

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NUCLEIC ACIDS AND PROTEIN SYNTHESIS IN CANCER CELL MITOCHONDRIA

II. AMINO ACID INCORPORATION INTO PROTEINS OF RAT LIVER AND HEPATOMA CELL MITOCHONDRIA*

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Since MCLEAN *et al.*(1) reported in 1958 that C^{14}-amino acid is incorporated into the proteins in the isolated mitochondrial systems, the mechanism of the amino acid incorporation has been investigated by many workers (2—9). On the other hand, the existence of nucleic acids, DNA and RNA, in mitochondria, has also been clarified (10—13). In 1959, REIS *et al.*(2) reported that the amino acid incorporation into the proteins in the isolated mitochondria is inhibited by the addition of cyanide. Recently, KROON (6) and BRONK (7), independently, have drawn the same conclusion that a high energy-intermediate in the respiratory chain phosphorylation is probably the energy source for the amino acid incorporation into mitochondrial proteins. It may be generally considered that mitochondria formation in the cells at the growth phase is probably more active than that at the stationary phase. It was reported, for example, that the rate of the amino acid incorporation into the proteins in regenerating liver mitochondria was higher than that in normal liver mitochondria.

There are many discrepancies, however, in the above conclusion (6, 7) to explain completely the energy supply mechanisms for the amino acid incorporation in mitochondria. It is necessary to maintain the isolated mitochondria in the reaction systems intact for the comparative studies between mitochondria isolated from different species on the rate of the amino acid incorporation.

The present experiments were conducted primarily on the energy reproducing system utilized for the amino acid incorporation into protein in mitochondria and on the rates of the amino acid incorporation into protein between mitochondria isolated from rat liver and from hepatoma

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(AH 130) cells. Observations on C^{14}-amino acid incorporation into proteins were carried out with mitochondria isolated from rat liver and hepatoma (AH 130) cells in the mild and physiological conditions. These conditions maintained mitochondria intact, and were scarcely affected by the following factors: the contamination of bacteria or ribosomal systems and the adsorption of C^{14}-amino acids onto mitochondrial protein.

MATERIALS AND METHODS

Isolation of mitochondria: Rat liver mitochondria were prepared by the method of HOGEBOOM (14). Rat ascites hepatoma (AH 130) mitochondria were isolated by modification of the preparation methods involving protease treatment (15—17).

Conditions of reaction mixture: The standard incubation mixture contained 174 μmoles sucrose, 10 μmoles potassium phosphate (K_2HPO_4) buffer (pH 7.5), 10 μmoles KCl, 7.5 μmoles MgCl, 100 mμmoles EDTA, 10 μmoles α-ketoglutarate or succinate, 4 μmoles ADP or ATP, 0.3 μc C^{14}-amino acid mixture prepared from algal protein hydrolyzate (10 μC/μmole) and 2 mg mitochondrial protein. The final volume was 0.8 ml and the reaction mixtures were incubated for 30 to 60 minutes at 25°C.

Extraction of proteins: The incorporation of C^{14}-amino acids was stopped by addition of 6.5 % trichloroacetic acid (TCA) containing 0.25 % sodium tungstate (pH 2.0). The precipitate obtained after centrifugation was resuspended in 5 % TCA containing 0.25 % sodium tungstate (pH 2.0), centrifuged, and these procedures were repeated 4 times. The precipitate was further washed with 3 % acetic acid on a millipore filter under suction.

Measurements of radioactivity: The final precipitate on a millipore filter was transferred to a nickel planchet and dried under an infra-red lamp. The radioactivity was determined in a thin-window gas-flow counter (2π gas-flow counter, Aloka FC-1E Nihon Musen K. K.) at infinite thickness. The results were expressed in terms of counts per minute of C^{14}-amino acid incorporated into mitochondrial proteins, from which the value at zero time was usually subtracted.

Determination of protein: Protein was determined by the method of LOWRY *et al.* (18).

Reagents: C^{14}-labelled amino acid mixture (hydrolyzate of algal protein) was the gift from Prof. A. TSUGITA, Laboratory of Molecular Genetics, Medical School, University of Osaka. All other reagents used were of analytical grade, and to avoid bacterial contamination in the solutions they were dissolved with fresh purified-distilled-water and stored at −20°C and thawed just before the use.

Abbreviations: EDTA, ethylenediaminetetraacetate; Tris, tris (hydroxymethyl) aminomethane.

RESULTS

Energy source for the C^{14}-amino acid incorporation in rat liver mitochondria:

The time course of the incorporation of C^{14}-amino acids into the proteins of rat liver mitochondria is shown in Fig. 1. The incorporation of amino acid was relatively rapid for the first 30 minutes and thereafter it increased constantly with lapse of time for 60 minutes in the presence of substrate. In order to eliminate bacterial effect as much as possible, the reaction was usually stopped at 30 or 60 minutes in these experiments.

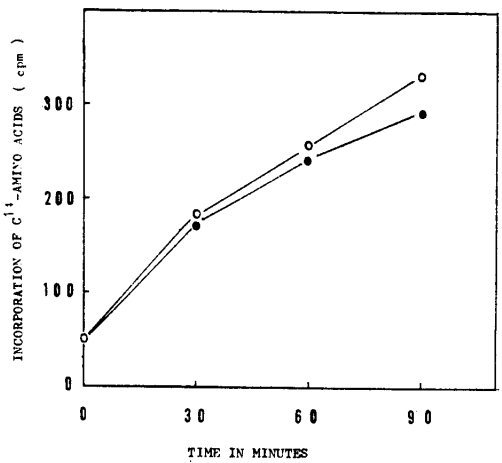

Fig. 1 Time course of the incorporation of C^{14}-amino acids in rat liver mitochondria. ○: Complete system contained 10 μmoles potassium phosphate (pH 7.4), 10 μmoles KCl, 7.4 μmoles $MgCl_2$, 100 mμmoles EDTA, 175 μmoles sucrose, 12.5 μmoles α-ketoglutarate, 4 μmoles ADP, 0.3 μC of C^{14}-amino acid mixture and 2.2 mg mitochondrial protein. ●: Endogenous system was the same incubation medium except omission of α-ketoglutarate and ADP. Reaction mixture was incubated at 25°C. Final volume was 0.8 ml.

Table 1 shows the effect of substrate on the C^{14}-amino acid incorporation in rat liver mitochondria. In the presence of α-ketoglutarate and ADP, the amino acid incorporation increased by about 40% on the average at 60 minutes. In contrast, the addition of succinate and ADP seemed to induce the decrease in the amino acid incorporation.

The question whether or not the amino acid incorporation was dependent on the oxidative phosphorylation (substrate level and/or respiratory chain phosphorylation) was investigated by adding various inhibitors. As shown in Table 2, the amino acid incorporation was inhibited by about 20% on the addition of 1×10^{-4} M 2, 4-dinitrophenol (DNP), and in the endogenous substrate system the addition of 1×10^{-4} M DNP did not affect significantly the amino acid incorporation. The amino acid incorporation

Table 1 Effect of substrate on the C^{14}-amino acid incorporation in rat liver mitochondria

Exptl. No.	Substrate	C^{14}-amino acid incorporation counts/min/tube		Relative radioactivity at 60 min
		30 min	60 min	%
1	Endogenous	164	195	100
	α-ketoglutarate+ADP	134	226	116
2	Endogenous	122	192	100
	α-ketoglutarate+ADP	130	203	106
3	Endogenous	155	175	100
	α-ketoglutarate+ADP	282	252	144
	α-ketoglutarate+ATP	262	301	172
	α-ketoglutarate+ADP+ATP	223	261	149
	Succinate+ADP+ATP	211	281	161
	α-ketoglutarate+ADP+ATP -mitochondria	17	14	8
4	Endogenous	153	201	100
	α-ketoglutarate	238	291	145
	α-ketoglutarate+ADP	204	380	189
	α-ketoglutarate+ATP	231	313	156
	ADP	127	143	71
5	Endogenous	315	505	100
	Succinate+ADP	298	360	71
6	Endogenous	221	364	100
	Succinate+ADP	188	404	111
7	Endogenous		745	100
	Succinate+ADP		528	71

Mitochondrial protein amount added per tube in these experiments was 2.2 mg to 2.6 mg. Incubations were carried out at 25°C. Final volume was 0.8 ml.

was not affected with oligomycin, whereas this incorporation was considerably inhibited with 1 mM KCN.

As illustrated in Table 3, the amino acid incorporation at the endogenous level was remarkably inhibited with 2 mM arsenite, and this inhibition was very weak in the presence of succinate plus ADP, in contrast to the cyanide inhibition.

From these data it is reasonable to assume that the energy source utilized for amino acid incorporation is mainly derived from substrate level phosphorylation.

C^{14}-amino acid incorporation in the mitochondria isolated from rat ascites

Table 2 Effect of inhibitors of respiratory chain phosphorylation on C^{14}-amino acid incorporation in rat liver mitochondria

Exptl. No.	Substrate	Inhibitor	C^{14}-amino acid incorporation counts/min/tube	% inhibition
1	Endogenous	–	195	–
	Endogenous	1×10^{-4}M DNP	205	− 5
	Endogenous	1×10^{-4}M DNP*	214	−10
	α-ketoglutarate+ADP	–	226	–
	α-ketoglutarate+ADP	1×10^{-4}M DNP	149	34
	α-ketoglutarate+ADP	1×10^{-4}M DNP*	175	23
2	Endogenous	–	662	–
	Endogenous	1×10^{-3}M KCN	232	65
3	α-ketoglutarate+ADP	–	203	–
	α-ketoglutarate+ADP	1×10^{-4}M DNP	183	10
	α-ketoglutarate+ADP	5 μg oligomycin	201	1
4	Succinate+ADP	–	404	–
	Succinate+ADP	1×10^{-4}M DNP	310	23
	Succinate+ADP	1×10^{-3}M KCN	113	72
5	Succinate+ADP	–	528	–
	Succinate+ADP	5×10^{-4}M DNP	135	74

Mitochondrial protein amount added per tube in these experiments was 2.2 mg to 2.6 mg. Incubations were carried out at 25°C for 60 minutes. Final volume was 0.8 ml.
*: Reaction mixture was preincubated with dinitrophenol at 25°C for 5 minutes before C^{14}-amino acid mixture was added.

Table 3 Effect of arsenite and cyanide on the C^{14}-amino acid incorporation in rat liver mitochondria

Inhibitor	Substrate	C^{14}-amino acid incorporation	% inhibition
		counts/min/tube*	
–	endogenous	745	–
2 mM As_2O_3	endogenous	323	57
2 mM As_2O_3	succinate+ADP	588	21
2 mM KCN	endogenous	342	54
2 mM KCN	succinate+ADP	329	56
–	succinate+ADP	656	–

* : 2.5 mg protein of rat liver mitochondria was added per tube, and final volume was 0.8 ml. Incubation was carried out at 25°C for 60 minutes.

hepatoma (AH 130) cells : Intact mitochondria were isolated from rat hepatoma (AH 130) cells and the rate of the C^{14}-amino acid incorporation

into the mitochondrial proteins was immediately estimated to compare with that in rat liver mitochondria. As shown in Table 4, the rate of the amino acid incorporation per mg protein of mitochondria does not differ significantly between rat liver and rat hepatoma (AH 130) cell mitochondria.

Table 4 Amino acid incorporation into protein fraction in rat liver or AH 130 cell mitochondria

Mitochondria	Reaction system	C^{14}-amino acid incorporation counts/min/tube	
		30 min	60 min
Rat liver Mt. *	α-KG+ADP	204	380
Rat liver Mt. *	α-KG+ATP	231	313
Rat liver Mt. *	endogenous	153	201
AH 130 cell Mt. *	α-KG+ADP	134	346
AH 130 cell Mt. *	α-KG+ATP	264	342
AH 130 cell Mt. *	endogenous	124	173
Rat liver Mt. **	α-KG+ADP	130	203
AH 130 cell Mt. **	α-KG+ADP	152	220

* : 2.3 mg protein of mitochondria was added per tube, and final volume was 0.8 ml.
** : 2.2 mg protein of mitochondria was added per tube, and final volume was 0.8 ml. Incubation was carried out at 25°C.
Abbreviation : α-KG, α-ketoglutarate

Ratios of volume, weight and protein content of rat hepatoma mitochondria as against those of rat liver mitochondrion : On the assumption that the volume of mitochondrion may be calculated approximately as an ellipsoid of revolution ($V_{Mt}=\frac{4}{3}\pi ab^2$, where "a" stands for long radius ; b for short radius), ultrathin sections of mitochondria were observed under an electron microscope. The long and short radii of each mitochondrion were estimated from the histograms (Figs. 2 and 3). The mean values of the long and short radii of rat liver and rat hapatoma (AH 130) mitochondria were 0.39 and 0.30 μ, and 0.28 and 0.18 μ, respectively. The ratio of the mitochondrial volume of rat liver to that of rat ascites hepatoma (AH 130) cells was about 4. The specific gravities of rat liver- and rat hepatoma cell-mitochondria were the same (1.18) and the ratio of water and protein contents appeared to be also at the same level in both mitochondria. Therefore, the ratio of the weight or protein amount per rat ascites hepatoma (AH 130) mitochondrion to that of rat liver mitochondrion may be calculated as one fourth.

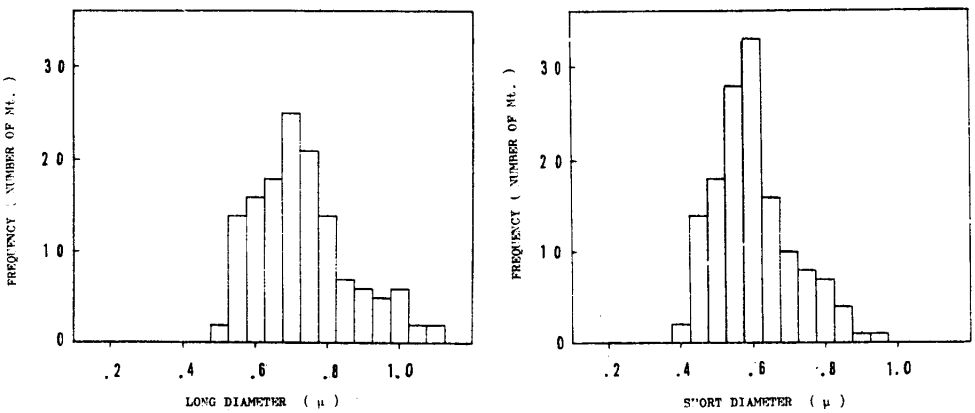

Fig. 2 Histograms of long and short diameters of rat liver mitochondria. Rat liver was fixed with potassium permanganate and sectioned. The ultrathin section of rat liver was observed by an electron microscope. Mean values of long and short diameters of mitochondrion were $0.77 \pm 0.14\,\mu$ and $0.60 \pm 0.11\,\mu$, respectively.

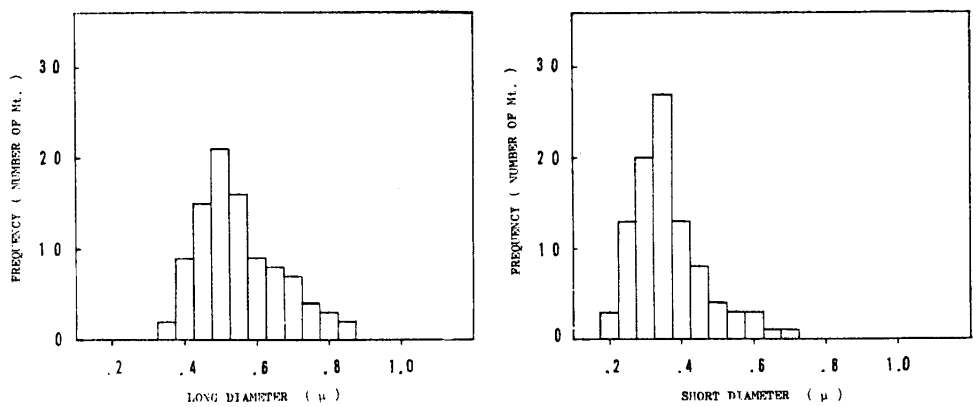

Fig. 3 Histograms of long and short diameters of rat ascites hepatoma (AH 130) cell mitochondria. Rat ascites hepatoma was fixed with potassium permanganate and sectioned. The ultrathin section of rat hepatoma was observed by an electron microscope. Mean values of long and short diameters of mitochondrion were $0.55 \pm 0.11\,\mu$ and $0.36 \pm 0.10\mu$, respectively.

DISCUSSION

Effects of microsomal and bacterial contaminations on the amino acid incorporation were scarcely observed by indirect determination in the present experiments (Table 5). Practically no incorporation of radioactive amino acids was observed in the reaction system where mitochondria were absent in the incubation mixture (Table 1). The time course of the amino acid incorporation was linear without a lag phase (Fig. 1). For the growth

Table 5 Effect of cyanide on C^{14}-amino acid incorporation in rat liver mitochondria and microsomal systems

Reaction system	KCN	C^{14}-amino acid incorporation counts/min/tube	% inhibition
Mt. *+succinate+ADP	–	528	–
Mt. *+succinate+ADP	1 mM	260	51.0
Mt. *+succinate+ADP+ATP	1 mM	191	74.0
Mic. **+succinate+ADP	–	413	–
Mic. **+succinate+ADP	1 mM	415	−0.5

* : 2.5 mg protein of rat liver mitochondria was added per tube.
** : 2.5 mg protein of rat liver microsome was added per tube.
Incubations were carried out at 25°C for 60 minutes. Final volume was 0.8 ml.

Table 6 Effect of aging on the C^{14}-amino acid incorporation in rat liver mitochondria

Preincubation (min)	Substrate	Inhibitor	C^{14}-amino acid incorporation counts/min/tube*		% inhibition at 60 min
–			30 min	60 min	
–	endogenous	–	315	505	–
17	endogenous	–	379	662	–
30	endogenous	–	434	710	–
17	endogenous	1 mM KCN	242	232	65
17	endogenous	1×10^{-4}M DNP	330	570	14
–	succinate+ADP	–	298	360	–
17	succinate+ADP	–	231	375	–
17	succinate+ADP	1 mM KCN	101	142	61
17	succinate+ADP	1×10^{-4}M DNP	224	381	−1

* : 2.5 mg protein of rat liver mitochondria was added per tube, and final volume was 0.8 ml. Preincubation and incubation were carried out at 25°C.

of most microorganisms a lag phase can naturally be expected. The amino acid incorporation in the endogenous substrate system was stimulated by aging for 17 to 30 minutes at 25°C but not in the case of the exogenous substrate system (Table 6). The adsorption of radioactive amino acids on mitochondria may only account for less than 10% of the total counts of the radioactive amino acids incorporated into mitochondria. Therefore, the possibility of these effects being responsible for the amino acid incorporation seems to be almost negligible.

Recently, KROON *et al.* (19) reported that there was not any significant difference in the radioactive amino acid incorporations into proteins between the sterile mitochondrial system and the non-sterile mitochondrial

system about which they had previously reported (6); namely, the bacterial contamination in the latter system scarcely affected the amino acid incorporations into mitochondrial proteins.

It has been proposed that the energy required for the amino acid incorporation into mitochondrial proteins is supplied through the process of oxidative phosphorylation (2, 3), since the amino acid incorporation into proteins is inhibited by anaerobic conditions or by the addition of cyanide or 2, 4-dinitrophenol. KROON (6) and BRONK (7) have reported independently that the energy source required for the amino acid incorporation into mitochondrial protein might be a high energy intermediate formed in the process of respiratory chain phosphorylation because the amino acid incorporation is insensitive to oligomycin, but is rather stimulated on the addition of thyroid hormone known as an uncoupler. In our experimental system the radioactive amino acid incorporation was also insensitive to oligomycin, and was scarcely or slightly inhibited on the addition of 1×10^{-4}M 2, 4-dinitrophenol, although concentrated 2, 4-dinitrophenol (5×10^{-4}M) considerably inhibited the amino acid incorporation into the mitochondrial protein.

There arise, however, many discrepancies in explaining the energy source required for the amino acid incorporation as a high-energy intermediate formed in the respiratory chain phosphorylation At first, the inhibitory effect of 2, 4-dinitrophenol is observed only at the high concentration which induced a decrease of respiration, fall-off during the incubation time. Secondly, thyroid hormone and 2, 4-dinitrophenol would also affect the permeability of the mitochondrial membrane, and the stimulation of amino acid incorporation with such agents cannot be explained so simply.

From our data we are of the opinion that the energy derived from the process in the substrate level phosphorylation would be required for the amino acid incorporation into mitochondrial protein; namely, the energy supply from the oxidative decarboxylation process of α-keto acids would be a rate-limiting step in the amino acid incorporation, and that the energy derived from the respiratory chain phosphorylation would have little effect on the amino acid incorporation into mitochondrial protein in at least the system of isolated mitochondria. Since the addition of ADP and succinate or ATP rather inhibits the amino acid incorporation, GTP generated in the process of oxidative decarboxylation would be utilized as an important energy source. It is also reasonable to assume that the stimulatory effect of the aging on the radioactive amino acid incorporation into mitochondrial proteins in the system of the endogenous respiratory substrates

might be due to the increase of the turnover rate in the oxidative decarboxylation as a result of respiratory release and/or GTP formed by the action of GTP-specific fatty acid thiokinase (20).

The rate of radioactive amino acid incorporation into proten in generating liver mitochondria is said to be twice faster than that in control liver mitochondria (8). It may be generally expected that the rate of amino acid incorporation into proteins is faster in those mitochondria isolated from cells at growing state. The rate of radioactive amino acid incorporation per mg protein, however, was at the same level between rat liver and hepatoma (AH 130) cell mitochondria. In this case, however, an interesting question may be raised here that the time required for the synthesis of membraneous protein in a mitochondrion of the hepatoma cells might fall several times short of that in rat liver cells.

Recently, GRAFFI *et al.* (21) reported that the incorporation of basic amino acid (arginine) into the mitochondrial protein is higher than that of neutral amino acid (leucine) in several cancer cell mitochondria.

The incorporated radioactive amino acid is known to be localized mainly in the insoluble protein fraction (22, 23), especially, in mitochondrial inner membraneous proteins (24). Relative to this problem we are now conducting investigation on differences in the properties of mitochondrial DNA and insoluble enzyme components of mitochondria between normal rat liver and rat hepatoma cells.

SUMMARY

The energy source required for the amino acid incorporation into mitochondrial proteins has been investigated and comparative study has also been made on the rate of the amino acid incorporation in rat liver and rat hepatoma cell mitochondria.

1. The incorporation of amino acid into the protein in intact mitochondria of rat liver increased by about 40% on the addition of α-ketoglutarate and ADP, but no significant increase in the amino acid incorporation was observed on the addition of succinate and ADP.

2. The incorporation of amino acids into mitochondrial proteins was remarkably inhibited by the addition of respiratory inhibitors (cyanide, DNP at a high concentration).

3. The amino acid incorporation into mitochondrial proteins was scarcely or slightly inhibited by the addition of DNP at the concentration of 1×10^{-4}M and insensitive to oligomycin (5 to 10 μg/ml).

4. The amino acid incorporation into the protein in the endogenous

substrate system of the mitochondria was considerably inhibited by the addition of arsenite, and this inhibition somewhat recovered on the addition of ADP plus succinate.

5. The rate of the amino acid incorporations between rat liver and hepatoma cell mitochondria was at the same level.

6. Discussions were made on the energy source required for the amino acid incorporation into mitochondrial proteins, on the rate of protein synthesis per mitochondrion isolated from rat liver- and hepatoma cells, and on the possibilities of contamination of bacteria or microsomes and of the adsorption of amino acids onto the mitochondria.

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