ON THE OSMOTIC BEHAVIOUR OF MITOCHONDRIA

by

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During the past years several investigators have described many techniques permitting the fractionation of the cells and the isolation from them of nuclei, mitochondria and microsomes in a state of high purity. Particular attention was directed to the study of mitochondria, which were recognized to be the site of several enzymic activities and particularly of those concerned with oxidation and with phosphorylative processes (HOGEBOOM *et al.*, BRACHET, SCHNEIDER, SWANSON, KIELLEY AND KIELLEY[1–8]).

The shape and the physical properties of mitochondria were studied by several investigators and it was established that the composition of the suspension medium has a great influence on the morphology of these cell particles. In fact mitochondria, which are in rodlet-form within the living cells, change their shape to spheres when they are isolated in the presence of electrolytes. It was also observed (HOGEBOOM[1,2], SCHNEIDER[4,5], HARMAN[9]) that, when mitochondria are isolated with 0.88 *M* sucrose, the transformation into the sphere form occurs on dilution of the suspension medium or on addition of electrolytes to it.

The behaviour of mitochondria in distilled water was also studied by CLAUDE[10,11], OPIE[12] and HARMAN[9]. CLAUDE has claimed that the suspension of mitochondria in distilled water causes absorption of water by the granula, with resulting decrease in density. Under darkfield illumination, mitochondria, which under normal conditions have a diameter of 0.5–2 micron, were observed to swell and increase in size enormously, reaching even 6 micron in diameter. CLAUDE affirmed that mitochondria break up spontaneously into units of smaller size and that this process is accelerated by hypotonic solutions. He claimed that microsomes are probably produced as a consequence of the disruption of mitochondria and concluded that mitochondria are surrounded by a semipermeable membrane.

A further argument supporting the theory of the presence of a semipermeable membrane in mitochondria was then suggested by CLAUDE AND FULLAM[13] and by DALTON *et al.*[14] with electronic microscopic observations.

OPIE (*l.c.*) has studied the osmotic behaviour of mitochondria within the cells. He observed swelling of mitochondria when the tissue sections were submitted to the action of distilled water and confirmed substantially the claims of CLAUDE on the presence of a semipermeable membrane. More recently HUENNEKENS AND GREEN[15] and HARMAN (*loc. cit.*) have cast some doubt on the real existence of a semipermeable membrane surrounding the mitochondrial body. HARMAN confirmed that mitochondria, which are originally rod-like in form, swell and shrink when suspended in hypotonic solutions. He observed, however, that neither distilled water nor 30% urea causes a release of soluble

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material into the supernatant, although they produce a great swelling of mitochondria.

This fact shows that mitochondria do not lose soluble proteins when suspended in distilled water or in hypotonic solutions. In addition, when mitochondria are suspended in media containing radioactive sodium and potassium ions, the radioactivity per ml of supernate and of mitochondrial residue is substantially the same. HARMAN claims that this fact excludes the presence of a semipermeable membrane similar to the one present in red cells, which is provided with a selective power on the penetration of radioactive ions.

A further contribution to the problem of the presence of a semipermeable membrane in mitochondria was given by DE DUVE *et al.*[16–18]. These authors have reported that 60% of the acid phosphatase of the cell is contained in mitochondria. When distilled water or salt solutions are added, the enzyme is released into the supernatant fluid. Sucrose protects against this loss of the enzyme from the particles. This fact seems to the authors an argument supporting the theory of the presence in mitochondria of a semipermeable membrane. Further argument for this theory was very recently suggested by WALKER[19], who showed that β-glucuronidase of liver mitochondria is released in the supernatant when these particles are suspended in distilled water.

An examination of the above-mentioned works shows that the problem of the presence of a semipermeable membrane in mitochondria is not clear. This paper is an attempt to contribute to its elucidation.

MATERIAL AND METHODS

Mitochondria were isolated from liver or kidney of white rats by differential centrifugation. The homogenates were prepared in refrigerated mortars from 1 g of tissue, which was diluted with 10 ml of 0.25 *M* sucrose in main experiments. 0.88 *M* sucrose, distilled water or 1 *M* NaCl solution was used instead of 0.25 *M* sucrose in some cases. Tissue debris, undamaged cells and nuclei were discarded by a first centrifugation at 1000 *g* for 15 min. A large portion of the mitochondria was then sedimented by centrifuging the residual fluid at 5000 *g* for 30 min. They were then resuspended in 0.25 *M* sucrose and resedimented for washings. The supernatant after the sedimentation of the mitochondria fraction, which contained some mitochondria of minor size, microsomes and soluble protein, was discarded. All operations were performed at 2° C.

Morphology, enzymic activities, and nucleic acid content of mitochondria suspended in media of different osmotic concentrations were particularly studied.

Morphology of mitochondria was studied with the Zeiss-Winkel phase contrast microscope. Mitochondria isolated in 0.25 *M* sucrose, in 0.88 *M* sucrose or in distilled water were suspended in media of different osmotic activity, at the following concentration: NaCl 0.075 *M* (hypotonic for red cells), 0.15 *M* (isotonic), 1 *M* (hypertonic); KCl 0.075 *M* (hypotonic), 0.15 *M* (isotonic), 1 *M* (hypertonic); sucrose: 0.125 *M* (hypotonic), 0.25 *M* (isotonic), 1 *M* (hypertonic); distilled water.

Succinoxidase, D-amino acid oxidase and lactic oxidase activities of isolated mitochondria were also studied in media of different composition, in order to see if the osmotic activity of the suspension medium has some influence on the enzymic activities. Some results in this field were obtained by HARMAN[8], who reported that cyclophorase activity of mitochondria is higher in 8.5% than in 17% sucrose. Since 0.067 *M* Na_2HPO_4–KH_2PO_4 buffer solution is usually employed as suspension medium for the routine determination of most enzymic activities, the results obtained in every experiment by suspending mitochondria in media of different osmotic activity were compared with those shown in 0.067 *M* phosphate buffer solution (which is hypotonic). In practice, the mitochondrial sediment obtained from 1 g of tissue was resuspended in 10 ml of 0.25 *M* sucrose and divided into 2 aliquots, each of 5 ml. Both aliquots were submitted to a new centrifugation in the same centrifuge for 30 min. The final pellets were then suspended in 3 ml of the 0.067 *M* phosphate buffer and in 3 ml of the other solution to be studied, respectively. For succinoxidase tests, 3 ml of the mitochondria suspension were placed in the main compartment of a Warburg vessel, the central well of which contained 0.2 ml of 30% KOH. 0.2 ml of 0.2 *M* sodium succinate were added to the suspension immediately before the beginning of the experiment and the first reading was made after 7–10 min necessary for temperature equilibration. Gaseous environment was air in most experiments, but 100% O_2 and 100% N_2 were used in some experiments. All experiments were performed at 38° C. Very similar

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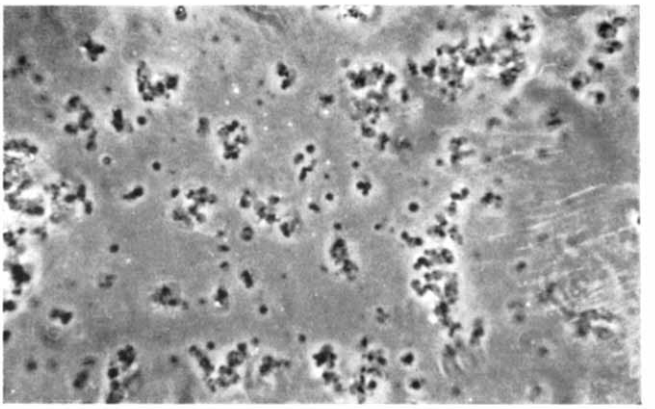

Fig. 1. Mitochondria suspended in 0.25 *M* sucrose. Phase Contrast, Ph 3 immersion objective, Magnification: 1000 × 3 × d.

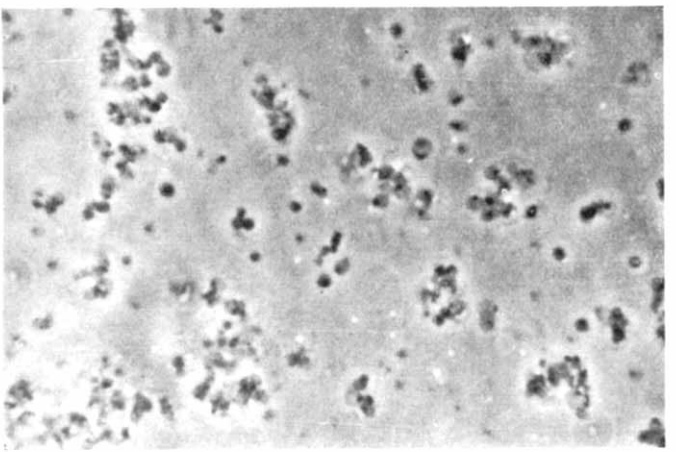

Fig. 2. The same image as Fig. 1, at higher magnification (1000 × 6 × d)

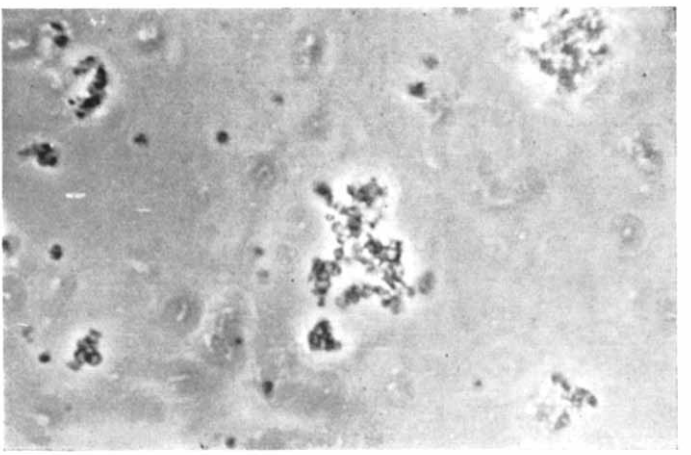

Fig. 3. Mitochondria suspended in 1 *M* NaCl solution for 8 h. Magnification: 1000 × 6 × d.

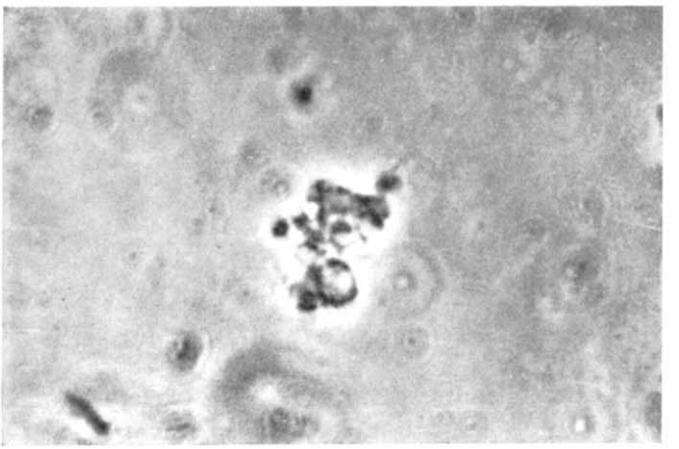

Fig. 4. Mitochondria suspended in distilled water for 8 h. Some of the most swelled elements. Magnification: 1600 × 6 × d.

procedures were employed for D-amino acid oxidase and for lactic oxidase, but 0.2 ml of 0.2 *M* DL-alanine or of 1 *M* sodium lactate were added instead of succinate.

Nucleic acid phosphorus was determined according to SCHMIDT AND THANNHAUSER[19].

RESULTS

Morphology

Mitochondria isolated with 0.25 *M* sucrose are sphere-shaped and their diameter is 0.5–2 micron approximatively. A more dense shadowy rind resembling a new moon can always be observed on one side of the sphere. This image is quite similar to the one described by CLAUDE, by ZOLLINGER[20] and by HARMAN.

When mitochondria are very freshly isolated, some rod-like forms can be observed, but the number of these forms is rapidly reduced with progress of time. 1 h after the isolation, only a few particles show a rod-like form. Some elements with intermediate forms between rods and spheres can be sometimes observed. Only spherical mitochondria can be observed in electrolyte solutions.

When mitochondria are isolated from homogenates with distilled water, they are always spherical and rod-like forms were never detected. The diameter of these mitochondria seemed to be slightly higher than that of mitochondria isolated with 0.25 *M* sucrose and many elements with diameter of 5–6 micron occurred in these preparations.

The mitochondrial sediment obtained from homogenates suspended in 0.88 *M* sucrose was constituted principally of rod-like elements, but a high percentage of spherical forms were also present. When this mitochondrial sediment was suspended in isotonic or in hypotonic solutions, the rod-like forms changed rapidly to spheres. When spherical mitochondria isolated with 0.25 *M* sucrose were suspended in hypotonic, isotonic or hypertonic media, no large modification in their morphology could be observed.

When 1 *M* sucrose solution was used as suspension medium, mitochondria appeared only very slightly reduced in size with respect to those suspended in 0.25 *M* sucrose. Rod-like form was never reassumed when spherical mitochondria were transferred to hypertonic (1 *M*) media.

Mitochondria suspended in electrolyte solutions appeared to be more agglutinated than those suspended in sucrose solutions.

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Suspension in distilled water of mitochondria isolated with 0.25 *M* sucrose did not produce further rapid swelling. Two hours after the suspension in distilled water, the particles appeared to be only faintly larger than the controls suspended in 0.25 *M* sucrose. The spherical form was conserved even 10 h after the suspension in water, but the number of forms with a large diameter increased with progress of time.

Enzymic activities

Effect of distilled water

In a first series of experiments the action of distilled water on the enzymic activities of mitochondria was studied. Washed mitochondria were suspended in 3 ml of distilled water (buffered by addition of 0.2 ml of 0.067 *M* phosphate buffer, pH 7.4). The homogeneous suspension was immediately transferred into a Warburg vessel and the experiment started. A control with the same amount of mitochondria suspended in 0.067 *M* phosphate was run at the same time.

As shown in Table I, suspension of mitochondria in distilled water produces a considerable increase of succinoxidase activity, while D-amino acid and lactic oxidases are inhibited to a large extent. The amount of the increase of succinoxidase activity is larger for kidney than for liver mitochondria. Also the amount of decrease of lactic and D-amino acid oxidases activities is larger for kidney than for liver mitochondria.

In an attempt to explain the nature of the effect of distilled water on succinoxidase activity of mitochondria, some experiments were made in which mitochondria were exposed to the action of distilled water for a longer period of time. For this purpose all operations were performed as described above, but mitochondria were incubated with distilled water for 15 min, 30 min, 1 h and 2 h. At the end of the incubation time, succinate was added and the experiment started. A non-incubated control was always established.

TABLE I

EFFECT OF DISTILLED WATER ON SUCCINOXIDASE, D-AMINO ACID OXIDASE AND LACTIC OXIDASE ACTIVITIES OF ISOLATED MITOCHONDRIA (THE DATA ARE EXPRESSED AS mmc O_2 CONSUMED IN 1 h AT 38° IN AIR BY MITOCHONDRIA CORRESPONDING TO 0.5 g OF FRESH TISSUE)

(S.D. = standard deviation; S.E. = standard error)

Enzyme	*Number of experiments*	*Values expressed as*	*Liver*			*Kidney*		
			Mitochondria suspended in 0.067 M phosphate buffer	*Mitochondria suspended in distilled water*	*Acceleration %*	*Mitochondria suspended in 0.067 M phosphate buffer*	*Mitochondria suspended in distilled water*	*Acceleration %*
Succinoxidase	7	mmc O_2 consumed	294.32	358.55	+ 21.8	230.84	314.26	+ 36.1
		S.D. ±	20.3	16.4	7.4	23.2	14.5	12.5
		S.E. ±	5.4	4.4	2.0	6.2	3.9	3.3
D-amino acid oxidase	3	mmc O_2 consumed	19.44	8.72	— 55.15	48.09	15.62	— 67.52
		S.D. ±	2.04	2.18	4.13	3.20	2.76	3.5
		S.E. ±	0.8	0.9	1.7	1.3	1.1	1.4
Lactic oxidase	3	mmc O_2 consumed	33.53	25.71	— 23.33	33.46	19.89	— 40.56
		S.D. ±	1.08	2.9	7.2	3.9	0.4	7.1
		S.E. ±	0.45	1.2	3.0	1.6	0.1	2.9

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The results which were reproduced in Table II show that, while non-incubated mitochondria show an increase of succinoxidase activity of 21.49%, incubation for longer periods of time reduces the extent of acceleration to 12.17% after 15 min., 7.5% after 30 min and 2.1% after 1 h, respectively. 2 hours of incubation results in a weak decrease of the activity (—4.16%).

TABLE II

EFFECT OF INCUBATION WITH DISTILLED WATER FOR VARIOUS EXTENTS OF TIME ON SUCCINOXIDASE ACTIVITY OF ISOLATED MITOCHONDRIA

(The data are expressed as mmc O_2 consumed in 1 h at 38° C)

	No incubation	*Incubation for 15 min*	*Incubation for 30 min*	*Incubation for 1 h*	*Incubation for 2 h*
Control	258.92	258.92	258.92	260.12	255.12
Distilled water	314.56	290.23	278.38	265.6	240.24
Acceleration %	+ 21.5	+ 12.17	+ 7.5	+ 2.11	— 4.16

A logical interpretation of this phenomenon might be the possible inactivation of the enzyme system, as a consequence of the disruption of an aliquot of mitochondria. This explanation would seem to be supported also by the results of CLAUDE, who reported that distilled water produces disruption of mitochondria with consequent production of particles of smaller size from them. In an attempt to verify this hypothesis, mitochondria were isolated from 1 g of rat liver and kidney, as usual. They were then resuspended in 15 ml of 0.25 *M* sucrose solution and divided in 3 aliquots, each of 5 ml. They were then again sedimented in a centrifuge. The first aliquot was suspended in 3 ml of 0.067 *M* phosphate buffer (pH 7.4); the second and the third aliquots were suspended in 3 ml of buffered distilled water. All three suspensions were incubated at 20° C for 30 min. The third aliquot was then again centrifuged at 5000 *g* for 30 min. The sediment was resuspended in 3 ml of 0.067 *M* phosphate buffer and both resuspended sediment and supernatant fluid were transferred into Warburg vessels.

Sodium succinate and KOH were added and succinoxidase activities of all three systems were determined parallelly.

As shown in Table III, only a very small amount of succinoxidase activity is released in the supernatant fluid and practically all the activity remains in the sediment. This fact does not agree with the hypothesis that mitochondria undergo disruption and fragmentation with consequent redistribution of enzymic activity when suspended in distilled water. Since fragmentation of mitochondria cannot be held responsible for the

TABLE III

EFFECT OF SUSPENSION IN DISTILLED WATER ON THE DISTINCTION OF SUCCINOXIDASE BETWEEN SEDIMENT AND SUPERNATE

Data expressed as	*Mitochondria suspended in 0.067 M phosphate buffer*	*Mitochondria suspended in distilled water*	*Distilled water supernatant*	*Distilled water sediment*
Mmc O_2 consumed	256.68	261.81	5.22	246.98
Acceleration %		+ 2		

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disappearance, following incubation, of the increase of succinoxidase activity, which can be observed immediately after suspension in distilled water, the hypothesis may be advanced that distilled water produces alterations of the mitochondrial surface, increases the permeability and facilitates the inactivation by oxidation processes of the enzymes contained in them.

Some experiments were then established with the purpose of verifying this hypothesis. Mitochondria isolated from 2 g of rat liver were divided into five identical aliquots, following the procedure outlined above. The first aliquot was then suspended in 0.067 *M* phosphate buffer solution and was retained as a control. The residual 4 aliquots were suspended in buffered distilled water. All the aliquots were then transferred into Warburg vessels. Succinoxidase activity of the first of 4 aliquots suspended in distilled water was immediately determined, as well as the activity of the buffer-suspended control. The 3 residual aliquots, which were also suspended in distilled water, were treated as follows: the first one was incubated with distilled water for 1 h and was retained as an incubated control; the second and third aliquots were similarly incubated with distilled water, but 100% O_2 and 100% N_2 were established respectively in their manometric systems. Succinoxidase activities of these samples were then determined.

Two of these experiments are recorded in Table IV. It results from these experiments that incubation in a 100% N_2 medium partially protects mitochondria succinoxidase against inactivation.

Incubation in a 100% O_2 medium seems, on the other hand, to accelerate the inactivation of the enzyme.

TABLE IV

EFFECT OF 100% O_2 AND OF 100% N_2 GASEOUS MEDIA ON THE DISAPPEARANCE OF THE ACCELERATION OF SUCCINOXIDASE ACTIVITY OF MITOCHONDRIA PRODUCED BY SUSPENSION IN DISTILLED WATER

(The data are expressed as mmc O_2 consumed in 1 h by mitochondria)

Experiment No.	*Buffer control*	*Distilled water*			
		Control	*1 h of incubation in air*	*1 h of incubation in 100% O_2*	*1 h of incubation in 100% N_2*
1	198.92	255.78	204.38	195.12	229.12
2	200.98	264.26	208.05	194.20	234.95

Effect of hypertonic solutions

The effect of hypertonic solutions on succinoxidase and D-amino acid oxidase activities of mitochondria was also investigated. Mitochondria isolated from 1 g of tissue with 0.25 *M* sucrose were divided into 2 identical aliquots, the first of which was suspended in 0.067 *M* phosphate buffer (pH 7.4), while the second one was suspended in a 1 *M* solution of the following substances: NaCl, KCl, Na_2HPO_4–KH_2PO_4 buffer, sucrose, mannitol, respectively. Sucrose and mannitol were chosen because of their non-electrolytic character. Results are recorded in Table V.

Strong inhibitions of succinoxidase and D-amino acid oxidase activities were produced by 1 *M* solutions. Liver succinoxidase was inhibited 91.5% by 1 *M* NaCl, 88.3% by KCl, 54.7% by 1 *M* phosphates, 53.7% by sucrose and 71.4% by mannitol.

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TABLE

EFFECT OF 1 *M* SOLUTIONS ON SUCCINOXIDASE AND D-AMINO

(S.D. = standard deviation

Enzyme	*Organ*	*Values expressed as*	*Mitochondria suspende*					
			NaCl			*KCl*		
			0.067 M buffer control	*1 M solution*	*Inhibition %*	*0.067 M buffer control*	*1 M solution*	*Inhibition %*
Succinoxidase	Liver	mmc O_2 consumed	286.52	24.21	91.5	317.87	37.18	88.31
		S.D. ±	9.92	7.7	2.8	18.7	9.1	2.2
		S.E. ±	4.1	3.2	1.1	9.3	4.5	1.1
	Kidney	mmc O_2 consumed	290.77	37.98	86.9	351.30	46.88	86.66
		S.D. ±	15.1	9.7	3.3	8.1	9.6	2.4
		S.E. ±	6.2	4.0	1.3	4.0	4.8	1.2
D-amino acid oxidase	Liver	mmc O_2 consumed	17.78	3.92	78.2	26.8	5.71	78.7
		S.D. ±	2.7	1.3	6.1	1.3	0.8	4.0
		S.E. ±	1.1	0.5	2.5	0.6	0.4	2.0
	Kidney	mmc O_2 consumed	69.45	5.64	90.08	119.06	7.93	93.34
		S.D. ±	23.3	2.8	7.8	10.8	0.8	0.1
		S.E. ±	9.7	1.1	3.2	5.4	0.4	0.05

Kidney succinoxidase was inhibited 86.9% by NaCl, 86.6% by KCl, 52.3% by phosphates, 65.6% by sucrose and 70.01% by mannitol. D-amino acid oxidase was inhibited: 78.2% in liver and 90.08% in kidney by 1 *M* NaCl; 78.8% in liver and 93.3% in kidney by KCl; 36.03% in liver and 40.26% in kidney by phosphates; 41.5% in liver and 39.7% in kidney by sucrose; 46.7% in liver and 38.4% in kidney by mannitol.

It results from these data that the inhibitions produced by KCl and NaCl 1 *M* solutions, which have very similar osmotic activities, are practically to the same extent; 1 *M* phosphate buffer and the non-electrolytes sucrose and mannitol, which have osmotic activities lower than 1 *M* NaCl and KCl, also exert lower inhibitions. A possible interpretation of the large difference between the inhibition values determined by 1 *M* NaCl and KCl and those determined by the other 1 *M* solutions may be, therefore, their different osmotic activities.

In an attempt to verify this hypothesis, a series of experiments was made in which the extent of inhibition of succinoxidase and D-amino acid oxidase activities of both liver and kidney mitochondria by NaCl and sucrose solutions of different molarities was checked.

As shown in Table VI, the extent of inhibition of succinoxidase activity decreases progressively with the decrease of the molarity. Acceleration values were obtained with 0.0185 *M* NaCl and with 0.037 *M* sucrose. D-amino acid oxidase, the inhibition of which decreases also with decreasing molarity of the solutions, showed a further strong inhibition with 0.0092 *M* NaCl and with 0.0185 *M* sucrose.

The inhibition values produced by NaCl solutions are approximately twice those produced by sucrose solutions. Since also the osmotic pressures exerted by NaCl solutions are approximately double those exerted by sucrose solutions of the same molarity,

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V

ACID OXIDASE ACTIVITIES OF ISOLATED MITOCHONDRIA

S.E. = standard error)

in 1 M solution of								
Na_2HPO_4–KH_2PO_4			*Sucrose*			*Mannitol*		
0.067 M buffer control	*1 M solution*	*Inhibition %*	*0.067 M buffer control*	*1 M solution*	*Inhibition %*	*0.067 M buffer control*	*1 M solution*	*Inhibition %*
306.62	138.75	54.75	307.64	142.22	53.74	295.39	83.50	71.74
11.5	3.06	0.7	8.6	2.1	2.0	0	4.8	1.6
5.7	1.5	0.3	4.3	1.05	1.0	0	2.4	0.8
332.8	158.73	52.31	317.21	108.95	65.6	336.51	100.92	70.0
0	6.8	2.0	17.1	0.9	1.6	8.5	0.6	0.7
0	3.4	1.0	8.5	0.4	0.8	4.2	0.3	0.3
37.63	24.05	36.03	37.36	21.84	41.51	36.35	19.87	46.7
2.3	0.8	1.6	6.7	4.0	0.1	2.7	1.4	0.1
1.1	0.4	0.8	3.3	2.0	0.05	1.3	0.7	0.05
140.04	83.4	40.26	80.18	48.68	39.77	138.66	85.64	38.24
7.4	5.0	6.7	0.6	2.3	1.7	16.0	16.3	4.8
3.7	2.5	3.3	0.3	1.1	0.8	8.0	8.1	2.4

TABLE VI

EFFECT OF THE MOLARITY OF THE SOLUTIONS OF NaCl AND SUCROSE ON SUCCINOXIDASE AND D-AMINO ACID OXIDASE ACTIVITIES OF LIVER AND KIDNEY MITOCHONDRIA OF WHITE RATS

(The results are expressed as percentages of inhibition or acceleration produced by solutions of different molarity). (+ = acceleration; — = inhibition)

Molarity of the solutions	*Liver*				*Kidney*			
	NaCl		*sucrose*		*NaCl*		*sucrose*	
	succinoxidase	*D-amino acid oxidase*	*succinoxidase*	*D-amino acid oxidase*	*succinoxidase*	*D-amino acid oxidase*	*succinoxidase*	*D-amino acid oxidase*
0.0092	+ 8.05	—38.78	—	—	+ 8.30	—53.45	—	—
0.0185	+ 4.85	— 6.03	+11.13	—34.91	+ 0.96	—23.77	+ 5.20	—56.25
0.037	—15.23	—21.15	+ 2.40	— 6.14	—15.30	—31.36	— 0.91	—27.07
0.075	—24.83	—25.54	—11.54	—33.36	—27.59	—38.19	—13.94	—34.94
0.15	—24.87	—45.82	—17.69	—19.11	—39.61	—54.01	—21.60	—28.80
0.25	—	—	—21.22	—16.87	—	—	—20.55	—32.15
0.30	—46.17	—47.33	—30.01	—20.91	—41.58	—66.40	—29.18	—37.83
0.60	—83.16	—75.17	—34.59	—39.19	—80.53	—81.17	—37.61	—55.06
0.80	—96.07	—86.30	—42.70	—42.11	—88.90	—94.34	—48.83	—57.64
1	—93.15	—81.03	—54.74	—41.56	—88.46	—94.33	—65.60	—59.72
2	—95.10	—96.36	—	—	—94.86	—97.34	—	—
Saturated	—97.5	—97.9	—89.42	—48.86	—96.1	—96.3	—87.95	—58.34

as a consequence of the dissociation of sodium chloride, these facts strongly agree with the hypothesis that the osmotic activity of the solutions is directly responsible for the difference of behaviour of the enzyme activities of mitochondria suspended in different media. Further experiments were made in order to see if the inhibition produced by

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1 *M* solutions is reversible. For this purpose, 1. mitochondria were isolated from homogenates in 1 *M* NaCl, and suspended as usual in 0.067 *M* phosphate buffer; 2. mitochondria were isolated as usual from 0.25 *M* sucrose homogenates and then suspended in 1 *M* NaCl and incubated for 1 h, 2 h and 3 h at 38° C or at 2° C. At the end of the incubation time they were again sedimented, suspended in 0.067 *M* phosphate buffer and their enzymic activity studied and compared with that of a non-incubated control.

It was seen with the first experiment that the activity of mitochondria isolated from hypertonic solutions is not lower than that of mitochondria isolated from 0.25 *M* sucrose. The second experiment, the results of which are represented in Table VII, showed that the temperature of incubation plays an important rôle. In fact, when this temperature was 2° C, only very slight decrease of activity was observed after 3 h of incubation. When it was 38° C, a permanent, very strong inactivation of enzymes occurred. Hypertonic solutions produce then, at 38° C, an irreversible inactivation of the enzymic systems of isolated mitochondria. The same enzymic activities are practically unaffected when the suspension in hypertonic solutions is made at low temperature for short periods of time.

TABLE VII

EFFECT OF INCUBATION AT 2° OR AT 38° C WITH 1 *M* SOLUTIONS ON SUCCINOXIDASE AND D-AMINO ACID OXIDASE ACTIVITY OF ISOLATED MITOCHONDRIA

Mitochondria isolated from	*Incubated with*	*Time of incubation*	*Temperature of incubation*	*Succinoxidase Inhibition %*	*D-amino acid oxidase Inhibition %*
Liver	NaCl 1 *M*	1 h	2°	4.99	—
Liver	NaCl 1 *M*	2 h	2°	7.64	—
Liver	NaCl 1 *M*	3 h	2°	9.41	—
Liver	NaCl 1 *M*	1 h	38°	61.15	—
Liver	NaCl 1 *M*	2 h	38°	84.69	—
Liver	NaCl 1 *M*	3 h	38°	86.33	—
Liver	NaCl 1 *M*	2 h	38°	89.56	66.59
Liver	NaCl 1 *M*	2 h	38°	89.74	60.37
Kidney	NaCl 1 *M*	2 h	38°	85.23	90.23
Kidney	NaCl 1 *M*	2 h	38°	91.32	84.96
Liver	sucrose 1 *M*	2 h	38°	88.47	45.17
Kidney	sucrose 1 *M*	2 h	38°	92.63	62.98

Release of nucleic acids from mitochondria suspended in media of different osmotic activity

Some experiments were made with the purpose of studying the behaviour of nucleic acids which are contained in mitochondria when these cell particles are suspended in media of different osmotic activities. 2 g of rat liver were weighed, homogenized, suspended in 10 ml of 0.25 *M* sucrose, and mitochondria were isolated as usual. They were then again suspended in 12 ml of 0.25 *M* sucrose and divided into 4 identical fractions, each of 3 ml. Mitochondria were then again sedimented by centrifugation and the sediments were resuspended in solutions of different osmotic activity and allowed to stand at 38° C for 1 h.

At the end of the incubation time, the samples Nos. 2, 3 and 4 were submitted to a new centrifugation at 5000 *g* for 60 min. Nucleic acids phosphorus of the unfractionated control (No. 1) and of the sediments and of the supernatant fluids of the samples Nos. 2, 3 and 4 were determined according to SCHMIDT AND THANNHAUSER with a Beckman Mod. DU spectrophotometer. Results, which are reproduced in Table VIII, show that

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TABLE VIII

EFFECT OF MEDIA OF DIFFERENT OSMOTIC ACTIVITY ON THE RELEASE OF NUCLEIC ACIDS FROM MITOCHONDRIA

(Technique of Schmidt and Thannhauser — Hydrolysis with 4 ml of 1 *N* KOH at 37° for 15 h — Phosphorus titration on 1 ml of the hydrolysate). (The values are expressed as γ of phosphorus)

S.D. = standard deviation; S.E. = standard error; RNA = ribonucleic acid; P = phosphorus

Source of P	*Nucleic phosphorus fractions*	*Values as γ of P per ml of hydrolysate*	*S.D.* ±	*S.E.* ±	*Range*
Unfractionated control	Total P	2.80	0.32	0.08	2.37–3.24
	RNA P	2.45	0.45	0.12	1.80–3.09
Distilled water sediment	Total P	1.84	0.05	0.002	1.80–1.88
	RNA P	1.65	0.06	0.002	1.57–1.69
Distilled water supernatant	Total P	0.95	0.08	0.003	0.90–1.04
	RNA P	0.74	0.04	0.002	0.70–0.78
0.25 *M* sucrose sediment	Total P	2.35	0.05	0.002	2.29–2.39
	RNA P	2.12	0.07	0.003	2.06–2.20
0.25 *M* sucrose supernatant	Total P	0.38	0.06	0.002	0.31–0.42
	RNA P	0.30	0.05	0.002	0.24–0.34
0.12 *M* sucrose sediment	Total P	2.15	0.17	0.07	2.05–2.34
	RNA P	1.86	0.13	0.05	1.71–1.95
0.12 *M* sucrose supernatant	Total P	0.59	0.08	0.03	0.53–0.69
	RNA P	0.50	0.18	0.07	0.36–0.62
0.15 *M* NaCl sediment	Total P	2.28	0.12	0.06	2.19–2.37
	RNA P	2.01	0.10	0.05	1.94–2.08
0.15 *M* NaCl supernatant	Total P	0.50	0.05	0.02	0.48–0.52
	RNA P	0.46	0.01	0.005	0.45–0.47
0.075 *M* NaCl sediment	Total P	2.04	0.04	0.02	2.02–2.07
	RNA P	1.86	0.13	0.06	1.77–1.96
0.075 *M* NaCl supernatant	Total P	0.72	0.1	0.05	0.65–0.79
	RNA P	0.57	0.1	0.05	0.50–0.65
1 *M* NaCl sediment	Total P	1.48	0.13	0.06	1.39–1.58
	RNA P	1.22	0.20	0.10	1.08–1.37
1 *M* NaCl supernatant	Total P	1.33	0.15	0.07	1.22–1.44
	RNA P	1.20	0.12	0.06	1.11–1.29
0.15 *M* KCl sediment	Total P	2.34	0.25	0.12	2.16–2.52
	RNA P	2.06	0. 7	0.08	1.94–2.19
0.15 *M* KCl supernatant	Total P	0.48	0.09	0.04	0.42–0.55
	RNA P	0.39	0.01	0.005	0.38–0.40
0.075 *M* KCl sediment	Total P	2.09	0.10	0.05	2.02–2.17
	RNA P	1.91	0.06	0.03	1.87–1.96
0.075 *M* KCl supernatant	Total P	0.72	0.10	0.05	0.65–0.79
	RNA P	0.55	0.07	0.03	0.50–0.60
1 *M* KCl sediment	Total P	1.50	0.08	0.04	1.44–1.56
	RNA P	1.29	0.01	0.005	1.28–1.30
1 *M* KCl supernatant	Total P	1.35	0.03	0.01	1.34–1.37
	RNA P	1.15	0.05	0.02	1.12–1.19

small amounts of DNA (desoxyribonucleic acid) can be detected in the mitochondrial fraction. This fact was reported also by CLAUDE (*l.c.*), but does not agree with the results of SCHNEIDER (*l.c.*), who could not find DNA in isolated mitochondria. Probably the presence of such small amounts of DNA may be accounted for by the disruption of some nuclei during the tissue homogenization and the release from them of chromatin threads provided with about the same sedimentation rate as mitochondria (VEGNI[22]). In fact, the DNA content of the mitochondria fraction is reduced progressively by washings; no thymine can be detected chromatographically in fourfold washed mitochondria (DIANZANI[23]).

These results agree with those reported by HARMAN, who was able to detect chromatin threads within the mitochondrial sediment by a stain reaction. The most important result seems, however, the observation that small amounts of nucleic acids are released in the supernate from mitochondria as a consequence of incubation. These amounts are larger for mitochondria which were suspended in distilled water or in hypotonic media than for those suspended in isotonic solutions (0.25 *M* sucrose, 0.15 *M* NaCl, 0.15 *M* KCl). They attain, however, their highest values when mitochondria are suspended in hypertonic (1 *M*) solutions. This fact seems not devoid of importance, when one considers that incubation in the same solutions inactivates succinoxidase and D-amino acid oxidase activities of mitochondria. This result agrees with the claims of MIRSKY[24], who described a method for the extraction of nucleoproteins from the cells based substantially on the solubility of these substances in 1 *M* NaCl solution.

DISCUSSION

From the experiments described in this paper, the conclusion can be drawn that the existence of a semipermeable membrane surrounding mitochondria is highly probable. The characteristics of this membrane seem, however, to be peculiar to mitochondria and quite different from those described for red cell membrane. In fact, the experiments carried out by phase contrast microscopy showed a large difference between mitochondria and red cells against hypertonic, isotonic and hypotonic solutions.

In fact, red cells are rapidly disrupted when suspended in distilled water, while mitochondria show only swelling and resistance to distilled water for times longer than 8 hours. In addition, distilled-water treated mitochondria do not resume their primitive shape when transferred into hypertonic media, and succinoxidase activity remains quantitatively in the sediment (mitochondria) when these cell particles are suspended in distilled water. It cannot be excluded that the change from rod to sphere form produces irreversible alteration of the structure of the mitochondrion. Some doubt might then be cast upon the actual existence of an active semipermeable membrane in mitochondria.

The results of the study of enzymic activities and of the release of nucleic acids from mitochondria suspended in media of different osmotic power show however that the existence of a membrane is highly probable. In fact, the osmotic activity of the medium influences strongly the enzymic activities, which are much lower in more concentrated than in more diluted solutions. Suspension in distilled water accelerates succinoxidase activity, but inhibits lactic oxidase and, more remarkably, D-amino acid oxidase. 1 *M* solutions bring about, on the contrary, strong inhibitions of all enzymic activities. The results of these experiments agree with the statement of HARMAN (*l.c.*)

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with regard to cyclophorase activity of mitochondria, which was found higher in 8.5% than in 17% sucrose.

The difference of behaviour between succinoxidase, D-amino acid oxidase and lactic oxidase activities of mitochondria treated with distilled water is difficult to explain, but it might be important to remember that both D-amino acid oxidase and lactic oxidase are concerned in activity with flavoenzymes.

The fact that the inhibitions produced by the concentrated solutions can be observed when both electrolytes and non-electrolytes are employed as suspension media, shows that the electrolytic character is not entirely responsible for the observed phenomena. Since the inhibition values obtained with NaCl solutions are about double those obtained with sucrose solutions of the same molarity, it seems evident that the osmotic pressure of the solutions, which is about double for the electrolyte NaCl with regard to sucrose, may be really an important factor of the phenomenon. In addition, resuspension in distilled water of mitochondria isolated or previously incubated with 1 *M* NaCl solution shows that the enzyme is not definitely inhibited when the cell particles are suspended in 1 *M* NaCl. These facts also agree with the hypothesis of the existence of osmotic properties in mitochondria.

Some other factors of lesser value may, however, be considered for the inhibition phenomenon. In fact, the inhibition percentages obtained with NaCl or with KCl are generally slight more than double those obtained with sucrose solutions. Another argument for the existence of an influence of ions on mitochondria is the morphological observation that these cell structures agglutinate spontaneously when suspended in electrolyte solutions, while they do not agglutinate when suspended in sucrose or mannitol solutions.

The nature of the substance in which mitochondria are suspended seems also to exert its own influence. In fact, mannitol solutions produced higher inhibitions on liver succinoxidase than sucrose.

The experiments of incubation of mitochondria with distilled water for long extents of time (3–8 hours) showed that neither a large number of mitochondria undergo disruption, nor are enzymes in an active form released from them. Only slight inhibition of succinoxidase, which is probably connected with an oxidation phenomenon, is determined by prolonged contact with distilled water. In fact, a gaseous medium of 100% O_2 accelerated and one of 100% N_2 reduced the rate of inactivation.

Nucleic acids are released from mitochondria into the supernatant fluids in amounts that are slightly higher when distilled water or hypotonic solutions are employed as suspension media than when isotonic fluids are used. The largest amount of nucleic acids is, however, released from mitochondria suspended in 1 *M* solutions, but this fact can probably be explained by the larger solubility of nucleoproteins in 1 *M* than in isotonic solutions (MIRSKY, *l.c.*). Release of nucleic acids from mitochondria cannot be considered responsible for the inhibition of enzymic activities that was observed in hypertonic media. In fact, resuspension in hypotonic media of mitochondria produced the return to normal oxidation values. In addition, the non-reversible inactivation of the enzymes that was produced after a long incubation with 1 *M* solutions at 38° C did not occur when incubation temperature was 2° C, although the amounts of nucleic acids released from mitochondria were practically the same at 2° and at 38° C. Perhaps an enzymic process is responsible for the non-reversible inactivation occurring at 38° C.

From the experiments described in this paper, the existence of a semipermeable

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membrane in mitochondria appears then to be highly probable. The osmotic characteristics of this membrane seem to be particularly efficient with regard to enzymic activities, also when mitochondria are changed from rodlet to sphere form. This fact may have a particular importance for the functioning of the cell. In fact, the statement that the enzymic activities are dependent upon the osmotic power of the solutions in which mitochondria are suspended may make it possible to understand why the respiratory activity of tissue slices are normally lower than that of homogenates and of isolated mitochondria. Since osmotic pressure within the cell seems to be higher than the osmotic pressure of blood, as was suggested by OPIE and as is shown also by the fact that mitochondria have a spherical shape in media which are isotonic for red cells, this fact may represent an important physiological mechanism for the regulation of the respiratory processes.

Further experiments on these problems are in progress.

SUMMARY

1. The action of media of different osmotic activity on the morphology, enzymic activities and nucleic acids content of isolated mitochondria was investigated.

2. Distilled water and hypotonic solutions produce swelling of mitochondria with consequent increase in size. Hypertonic solutions produce only a slight decrease of size. Resuspension of previously distilled water-treated mitochondria in hypertonic solutions does not produce the return to the rod-like shape that is peculiar for mitochondria isolated from hypertonic solutions.

3. Succinoxidase activity of isolated mitochondria increases by dilution of the medium and reaches maximal values in distilled water. Hypertonic solutions inhibit strongly. Intensity of inhibition seems to be dependent upon the osmotic activity of suspension media.

4. Incubation with distilled water produces disappearance of the acceleration of succinoxidase activity determined by distilled water. Disappearance of the acceleration is enhanced by a gaseous environment of oxygen and decreased by a nitrogen environment.

5. Incubation for 2 hours in hypertonic solutions produces inactivation of succinoxidase at 38° C. Inactivation does not occur at 2° C.

6. Suspension of mitochondria in distilled water does not produce redistribution of succinoxidase activity, which remains bound to the mitochondrial sediment.

7. D-amino acid oxidase and lactic acid oxidase of mitochondria are inhibited by suspension in distilled water.

8. Nucleic acids are released from mitochondria as a consequence of incubation for 1 h at 38° C. Maximal values of release were obtained with hypertonic (1 *M*) solutions, but hypotonic solutions and distilled water gave also higher values than isotonic media.

9. The results are discussed, particularly with regard to the question of the existence of a semipermeable membrane in mitochondria.

RÉSUMÉ

1. L'action de milieux d'activités osmotiques différentes sur la morphologie, les activités enzymatiques et la teneur en acides nucléiques de mitochondries isolées, a été étudiée.

2. L'eau distillée et les solutions hypotoniques produisent un gonflement des mitochondries, dont la taille augmente. Des solutions hypertoniques ne produisent qu'une légère diminution de la taille. La mise en suspension, dans des solutions hypertoniques, de mitochondries préalablement traitées par l'eau distillée, n'est pas suivie d'un retour à la forme en batonnets, particulière aux mitochondries isolées de solutions hypertoniques.

3. L'activité de la succinoxydase des mitochondries isolées augmente avec la dilution du milieu, et est maximum dans l'eau distillée. Les solutions hypertoniques la diminuent fortement. L'intensité de cette inhibition dépend de l'activité osmotique du milieu de suspension.

4. L'incubation dans l'eau distillée entraîne la disparition de l'accélération de l'activité succinoxydasique, produite par l'eau distillée. Ce phénomène est plus marqué en atmosphère d'oxygène qu'en atmosphère d'azote.

5. L'incubation pendant 2 heures à 38° C, en solution hypertonique, inactive la succinoxydase. Cette inactivation n'a pas lieu à 2° C.

6. La mise en suspension des mitochondries dans l'eau distillée n'entraîne pas de redistribution de l'activité succinoxydasique, qui reste liée aux mitochondries.

7. La D-aminoacide oxydase et la lactique acide oxydase des mitochondries, sont inhibées par la mise en suspension dans l'eau distillée.

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8. Des acides nucléiques sont libérés des mitochondries après incubation pendant 1 heure à 38° C. La libération est maximum dans les solutions hypertoniques (1 *M*) mais elle est plus forte dans des solutions hypotoniques et l'eau distillée que dans un milieu isotonique.

9. Les résultats sont discutés, particulièrement du point de vue de l'existence d'une membrane semi-perméable des mitochondries.

ZUSAMMENFASSUNG

1. Es wurde die Wirkung von Medien mit verschiedener osmotischer Aktivität auf die Morphologie, die enzymatische Aktivität und den Nucleinsäuregehalt isolierter Mitochondrien untersucht.

2. Destilliertes Wasser und hypotonische Lösungen erzeugen ein Schwellen der Mitochondrien, das mit einen Zunahme der Grösse verbunden ist. Hypertonische Lösungen erzeugen nur eine geringe Abnahme der Grösse. Suspendiert man die zuerst mit destilliertem Wasser behandelten Mitochondrien wieder in hypertonischen Lösungen, so bildet sich nicht die stäbchenähnliche Form zurück, die den aus hypertonischen Lösungen erhaltenen Mitochondrien eigen ist.

3. Die Succinoxydaseaktivität isolierter Mitochondrien nimmt mit der Verdünnung des Mediums zu und erreicht in destilliertem Wasser Maximalwerte. Hypertonische Lösungen wirken stark hemmend. Die Intensität der Hemmung scheint von der osmotischen Aktivität des Suspensionsmediums abhängig zu sein.

4. Bei Inkubation mit destilliertem Wasser verschwindet die durch destilliertes Wasser bewirkte Erhöhung der Succinoxydaseaktivität. Das Verschwinden dieser Erhöhung wird in einer Sauerstoffgasphase noch befördert und in einer Stickstoffphase verlangsamt.

5. Zweistündige Inkubation in hypertonischen Lösungen erzeugt eine Inaktivierung der Succinoxydase bei 38°. Die Inaktivierung tritt nicht bei 2° ein.

6. Eine Suspension von Mitochondrien in destilliertem Wasser erzeugt keine Wiederverteilung der Succinoxydaseaktivität, sie bleibt an den Mitochondrienniederschlag gebunden.

7. Die D-Aminosäureoxydase und die Milchsäureoxydase der Mitochondrien werden durch Suspension der Mitochondrien in destilliertem Wasser gehemmt.

8. Als eine Folge der einstündigen Inkubation bei 38° werden Nucleinsäuren aus den Mitochondrien in Freiheit gesetzt. Maximalwerte der Freisetzung werden mit hypertonischen Lösungen (1 *M*) erhalten, aber hypotonische Lösungen und destilliertes Wasser geben ebenfalls höhere Werte als isotonische Medien.

9. Die Ergebnisse werden teilweise im Hinblick auf die Frage der Existenz einer semipermeablen Membran in den Mitochondrien besprochen.

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