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STUDIES ON THE MECHANISM OF OXIDATIVE PHOSPHORYLATION

III. PHOSPHORYLATING PARTICLE TYPES FROM BEEF HEART

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Previous reports from this laboratory have indicated the presence of several particle types in crude mitochondrial suspensions, prepared from beef heart, which are capable of catalysing oxidative phosphorylation[1]. These crude suspensions of mitochondria, readily prepared in large amounts from slaughterhouse material[2], can be further fractionated to yield preparations which are very active both with respect to oxidation and to phosphorylation. The early results along these lines have been briefly mentioned[1].

It is the purpose of this communication to describe the method of isolation and the enzymic properties of these particles. A preliminary report on this work has been presented earlier[3].

METHODS

Assay

The assay employed for measuring oxidative phosphorylation has been partially modified from that previously reported[1]. Oxygen uptake was measured by the standard Warburg technique at 30°C with an equilibration period of six minutes, during which time the oxygen uptake was assumed to be linear and equivalent to that in the subsequent six minute period. In a final volume of 3 ml the following components were added: three to eight mg particle protein, 20 to 80 μmoles phosphate

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References p. 88.

pH 7.2, 5 μmoles ATP*, 0.5 mg hexokinase, 5 μmoles $MgCl_2$, 100 μmoles glucose and 0.25 *M* sucrose to volume. Where indicated, the following were added: 20 μmoles pyruvate, 20 μmoles malate, 50 μmoles succinate, 50 μmoles D (—) β-hydroxybutyrate, 10 μmoles *d*-isocitrate, 1 μmole DPN, 0.5 μmole TPN, 30 μmoles α-ketoglutarate, and 50 μmoles L-glutamate. In the assay with ferrocyanide as electron donor, in addition to the basal components, the following were added: 5 μmoles ferricyanide, 20 μmoles ascorbate, and 15 μmoles $MgCl_2$. The particle preparations were stored routinely at —20°C at a concentration of 20 to 30 mg protein per ml and were thawed in cooled tap water shortly before use. The results to be described were obtained with unfrozen preparations unless otherwise mentioned.

Fractionation of mitochondrial suspension

Mitochondrial suspensions, prepared in the same manner as described previously by CRANE *et al.*,[2] are suspended in six to eight volumes of 0.25 *M* sucrose which is 0.01 *M* with respect to Tris (pH 7.5). The suspension is centrifuged for ten minutes at 17,000 r.p.m. in the No. 30 rotor of the Spinco preparative centrifuge. The sediment is composed principally of two distinct layers: a faster sedimenting layer designated hereafter as "heavy" particles and a slower sedimenting one designated as "light" particles. The light particles can be sloughed off and separated from the heavy fraction by means of a stirring rod. The heavy fraction is then resuspended in the Tris-sucrose solution and the above procedure repeated until no more light particles can be separated. Usually after the third centrifugation, the heavy fraction is almost completely free of light particles. The same procedure is followed to obtain a light fraction uncontaminated with heavy particles. These particles are isolated in roughly equal amounts from the starting material. The difference in the sedimentation properties between heavy and light particles is accentuated essentially by the pH of the medium and not by the nature of the buffer. At lower pH values the separation of the particle types is very poor; higher pH values affect good separation, but also damage the enzymic activity of the particles.

RESULTS

Electron microscope studies on these and related particles have been carried out by Professor HANS RIS of this university in collaboration with Drs. ZIEGLER, LINNANE, AND GREEN of this laboratory and will be reported separately. For the purposes of this communication, suffice it to say that none of the mitochondrial particles is morphologically identical with those seen in the heart muscle slices. The heavy particles are similar to the native mitochondria in that they are very osmiophilic whereas the light particles are not. The heavy particle fraction is also much more homogeneous than the light fraction.

The data in Table I indicate the typical oxidation and phosphorylation rates observed with heavy and light particles in presence of each of the several conventional substrates. These values were obtained with heavy and light particles isolated from the same suspension of starting material. With respect to substrate oxidations which are dependent on pyridine nucleotide, the heavy particles are two to three times more active than the light particles. The P/O ratios observed with the heavy particles are only slightly higher than those with the light particles and approach the assumed theoretical values.

The most striking enzymic difference between light and heavy particles lies in succinate oxidation and phosphorylation. The light particles exhibit a high rate of uncoupled oxidation of succinate, whereas the heavy particles always catalyze a lower rate of oxidation associated with P/O ratios very close to 2.0. These differences between the two particle types with respect to succinate oxidation and phosphorylation are qualitatively paralleled to those which are observed in the case of ferrocyanide

* The following abbreviations will be used in the text: adenosine triphosphate, ATP; diphosphopyridine nucleotide, DPN; triphosphopyridine nucleotide, TPN; electron transport particle, ETP; phosphorylating electron transport particle, PETP; trishydroxymethyl aminomethane, Tris.

References p. 88.

TABLE I

OXIDATIVE PHOSPHORYLATION IN HEAVY AND LIGHT PARTICLES

Expt.	Substrate	Oxidation rate*		Phosphorylation rate**		P/O	
		Heavy	Light	Heavy	Light	Heavy	Light
1.	Pyruvate + Malate	0.241	0.107	0.721	0.293	2.99	2.74
	β-Hydroxybutyrate	0.045	0.018	0.116	0.050	2.58	2.75
	α-Ketoglutarate	0.110	0.054	0.355	0.145	3.23	2.69
	Glutamate	0.177	0.086	0.372	0.188	2.10	2.19
	Isocitrate	0.003	0	0	0	—	—
	Isocitrate + DPN + TPN	0.043	0.079	0.055	0.072	1.28	0.91
	Succinate	0.077	0.115	0.149	0.056	1.93	0.36
	Ferrocyanide	0.022	0.050	0.019	0.006	0.88	0.13
2.	Pyruvate + Malate	0.248	0.107	0.687	0.224	2.77	2.12
	Malate	0.011	0.009	0.019	0.011	—	—

* μatoms oxygen per minute per mg protein.
** μmoles phosphate per minute per mg protein.

oxidation and phosphorylation. Ferrocyanide oxidation was carried out under the conditions used by JACOBS[4] for rat liver mitochondria. The values obtained should not be considered as maximal since no study was made to see whether these conditions are optimal for the heart system. Despite the fact that very rapid oxidation of pyruvate + malate ensues in the absence of added pyridine nucleotides, as previously described by us and others[1,5,6] almost no isocitrate oxidation can be measured unless the system is supplemented with pyridine nucleotide. This was true for both heavy and light particles. Isocitrate oxidation in the supplemented system is faster with the light particles and is accompanied, with both particle types, by poor phosphorylation. The P/O ratios obtained have never been very much above 1. It is possible that this phosphorylation is associated only with the subsequent oxidation of α-ketoglutarate.

It should be mentioned that the assay method routinely used was chosen for reproducibility rather than for accurate determination of maximal P/O ratios. The methods used were convenient, gave consistent results, and were used principally for the purpose of making precise comparisons between different preparations or of the same preparation as a function of time of storage. However, some problems in measuring oxidation rates and accurate P/O ratios were recognized. The heavy particles, for example, could only be assayed in a narrow range of protein concentration. When the concentration of protein was about three to five mg per flask, the oxidation rates were directly proportional to protein concentration. At lower concentrations of protein, however, the specific oxidation rate decreased in all preparations tested, the magnitude of the decrease varying with the preparation. This effect could be either partially or completely reversed, in all cases, by the addition of bovine serum albumin (20 mg per flask). At higher protein levels oxygen seemed to be a limiting factor, and either very high levels of phosphate would be required or the reaction would have to be terminated in a matter of a few minutes—a procedure which leads to technical errors. For example, when the oxidation of pyruvate plus malate was measured, it was observed that, during the six minute temperature equilibration period, less phosphate was esterified than in each subsequent six minute period wherein the same amounts of phosphate were esterified. This would indicate that under the conditions of the routine method employed, *i.e.* where three to five mg protein were used, the oxygen uptake is slightly

References p. 88.

overestimated. However, under the conditions used, the protein level was in the linear response range, and the oxygen uptake and the phosphate esterification proceeded linearly with time after the initial equilibration period. These constant technical errors would amount to less than 20 % where high protein levels (eight to ten mg per flask) were used, and would increase with the activity of the particles. Thus, it was observed that, even at three to five mg protein per flask, whenever the oxidation rate of pyruvate + malate was equal or higher than 0.3 μatoms/mg/min, the P/O ratio rarely exceeded the value of 2.5.

Stability of heavy mitochondrial particles

The stability of the crude mitochondrial suspensions has been briefly mentioned[1]. The data presented in Table II show the effect of extended storage in the deep-freeze on oxidative phosphorylation with two preparations of heavy particles. It can be seen that oxidation and phosphorylation rates in presence of pyruvate + malate remained practically unchanged even after 16 days of storage at —20°C, and declined only slightly after three months of storage. Similar results were obtained with other substrates of DPN-linked oxidation reactions. The succinoxidase system behaves quite differently on storage. In this case the effect was an increase in the rate of oxidation and a decrease in both the rate of phosphorylation and the P/O ratio. It may be added that in numerous particle preparations, which have been handled in different ways, the P/O ratio for succinate has consistently been observed to be inversely related in a strict manner to the rates of oxidation.

The effect of storage on the succinate system of the heavy particle appeared to be that of transforming the particle into one with the enzymic characteristics of the light particles already described, that is, a high oxidation rate with a low P/O ratio. The following experiment was therefore performed. Heavy particles were prepared by repeated centrifugations in the buffered sucrose medium until the preparation was free of light particles. After storage at deep-freeze temperature for one or two days, the particles were again subjected to differential centrifugation in the Tris-sucrose medium Two distinct layers of sediment appeared which were separated and assayed. As

TABLE II

EFFECT OF STORAGE AT — 20°C ON THE STABILITY OF THE HEAVY PARTICLES

Preparation	*Days at —20° C*	*Substrate*	*Oxidation rate**	*P/O*
I	0	Pyruvate + Malate	0.283	2.22
	2	Pyruvate + Malate	0.283	2.15
	16	Pyruvate + Malate	0.303	2.09
	92	Pyruvate + Malate	0.219	1.95
	0	Succinate	0.072	1.97
	2	Succinate	0.090	1.30
II	0	Pyruvate + Malate	0.286	2.32
	1	Pyruvate + Malate	0.283	1.97
	2	Pyruvate + Malate	0.272	2.08
	87	Pyruvate + Malate	0.232	1.85
	0	Succinate	0.059	2.01
	7	Succinate	0.088	1.09

* μatoms oxygen per minute per mg protein.
Aliquots of each preparation were thawed only once and assayed at the times given above.

References p. 88.

indicated in Table III, the upper layer corresponds very closely to the light particles in activity characteristics for succinate. The fact that the lower layer had no better activity than the starting material might be attributed to a deleterious effect resulting from the handling of aged mitochondria during refractionation. The effect of aging upon mitochondrial integrity will be discussed in a separate communication[7]. It may be concluded, therefore, that the high uncoupled rate of succinate oxidation is associated in this case with a change in the physical state of the particles as evidenced by a slower sedimentation rate.

TABLE III

PRODUCTION OF "LIGHT" PARTICLES AFTER STORAGE AND REFRACTIONATION OF HEAVY PARTICLES

Experiment	*Age and treatment of preparation*	*Oxidation rate*	*P/O*
I	0	0.057	1.97
	1 Day	0.068	1.18
	1 Day refractionated:		
	Lower layer	0.062	1.07
	Upper layer	0.114	0.65
II	2 Days	0.055	1.59
	2 Days refractionated:		
	Lower layer	0.051	1.57
	Upper layer	0.125	1.00

Substrate: Succinate. Other details in text.

DISCUSSION

It is clear that in the large scale preparation of beef heart mitochondria, the use of the blendor leads to the formation of particles which are not morphologically identical with those seen in sections of fresh heart muscle. These particulate suspensions of mitochondrial origin can be fractionated to yield a number of particle types with different sedimentation properties and with different, distinctive capacities for oxidation and phosphorylation. The bulk of the material is composed of the heavy and light material; in addition, two smaller particle types, ETP[2] and PETP[1,8] have been isolated. With the exception of uncoupled oxidation of succinate catalyzed by the light particles, the heavy and light particles carry out oxidative phosphorylation at rapid rates. PETP qualitatively resembles the light particles in that succinate oxidation is uncoupled whereas the DPN-linked oxidations, although proceeding at low rates, are associated with high P/O ratios. Finally, ETP can only oxidize DPNH and succinate at significant rates and has lost all capacity for oxidative phosphorylation.

Increases in the rate of uncoupled succinate oxidation have been observed previously to occur upon aging or other damage to heart particles.[9] It is seen in the experiments herein reported that this phenomenon is associated with a change in the physical properties of the material. Our previous failures to obtain consistently high P/O ratios for succinate with crude particle suspensions can now be understood and remedied by a simple fractionation. There may possibly be a similar reason for the low succinate P/O ratios reported for heart mitochondria isolated from other animals[9].

It has been indicated that the complete citric acid cycle is operative during the oxidation of pyruvate plus malate in the absence of added cofactors.[1] The results

References p. 88.

obtained in this investigation also support this view. However, externally added isocitrate is not oxidized by these preparations of heart mitochondria unless the system is supplemented with added pyridine nucleotides. Isocitrate oxidation, in the supplemented system, is not only considerably slower than the over-all rate for citric acid cycle as observed with pyruvate plus malate, but also this oxidation is largely uncoupled. These results imply that the slow oxidation of externally added isocitrate induced by pyridine nucleotide is carried out by an enzyme system different from that operative in the coupled oxidation of pyruvate plus malate and may be a consequence of the partial derangement of structure produced in the preparation of these particles. If the non-reactivity of heart mitochondria toward externally added isocitrate parallels the *in vivo* situation, it is significant that no extra-mitochondrial system has yet been described which generates isocitrate. Thus mitochondria may never be faced with the necessity for dealing with externally generated isocitrate.

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SUMMARY

Crude mitochondrial suspensions, prepared on a large scale from beef heart, have been fractionated by centrifugation in a buffered medium. Two principal fractions, heavy and light, have been isolated, and some of their properties with respect to the oxidation of citric acid cycle substrates and the accompanying phosphorylation have been studied. The heavy fraction, which resembles intact mitochondria much more closely than the light, catalyzes the oxidation of these substrates very efficiently with P/O ratios quite close to the assumed theoretical values. These preparations can be conveniently stored in the deep-freeze for long periods of time without considerable loss of activity.

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