Some Chemical Components of Mitochondria and Microsomes from Germinating Castor Beans

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Increasing interest in the biochemical activities of mitochondria from plants has made it desirable to have more knowledge of the chemical composition of such particles. We have, accordingly, extended our investigation of the highly active mitochondrial fraction from the endosperm of germinating castor bean (Beever & Walker, 1956; Walker & Beever, 1956) by studying some of the components of particulate preparations from this material. A considerable amount of analytical work has been done with particles of animal origin, but the data on biochemically active particles from plants is still rather meagre (see reviews by Goddard & Stafford (1954), and Hackett (1955)). Previous work is discussed in papers by Martin & Morton (1956*a, b*), who have made thorough examinations of the chemical composition of mitochondria and microsomes derived from silver-beet petiole and from wheat roots. Another recent contribution provides data on the ribonucleic acid (RNA) and nitrogen contents of active mitochondria and microsomes from peanut (Kmetec & Newcomb (1956)).

In the next paper (Akazawa & Beever, 1957), there are shown some very striking changes in the amount and intrinsic activity of the mitochondria derived from the endosperm as the castor-bean seedling grows to the 8 days stage, when the endosperm has been depleted. It was therefore of some interest to see if there were differences in the composition of the particles derived from seedlings of different ages. The information so obtained allows some conclusions to be drawn about the process of mitochondrial production and particularly about the decline in efficiency which characterizes the mitochondria from the ageing endosperm.

Osmotic phenomena are of some importance in determining the behaviour of plant mitochondria, as they are in those from animal tissues, and Laties (1953) and Farrant, Robertson & Wilkins (1953) have investigated aspects of the osmotic behaviour of plant mitochondria. These observations have been supplemented here by data on the percentage dry weight of mitochondrial particles and the changes in this value under a variety of experimental conditions.

MATERIALS AND METHODS

The culture of castor-bean seedlings and the preparation of mitochondrial suspensions were carried out as described previously (Beever & Walker, 1956), with the following changes: (a) variety Cimmarron was used, since the supply of U.S. 74 failed; (b) blending for 15 sec. in a chilled blender was substituted for manual grinding with pestle and mortar; (c) the phosphate concentration in the medium was reduced to 0.05 M. In this paper we refer to the washed mitochondrial suspension so obtained as 'crude mitochondria'. In preparing extracts a number of seeds, sufficient to give 40 g. of material, was used; the testa, root and shoot were discarded and in seedlings older than 6 days the cotyledons were also removed from the endosperm material. The crude mitochondria were in some cases separated into two components, which are designated as M_1 and M_2 ; the sediment M_1 was obtained by centrifuging for 10 min. at 1000 g and the resulting supernatant was exposed to 20 000 g for 20 min. to provide sediment M_2 . These fractions were resuspended in measured amounts of medium before analysis.

A further fraction, which will be designated 'microsomes', was obtained from the supernatant after removal of the mitochondria by centrifuging at 56 000 g for 60 min. in a Spinco ultracentrifuge. The usual precautions were taken to ensure cold conditions (0–5°) during the various manipulations. The fractions were analysed individually for the components listed below, and for oxidative activity where this was appropriate.

Most of the results are expressed on a nitrogen basis to facilitate comparison with those obtained in the extensive work on fractions from liver. In addition, in order to arrive at a picture of the interrelationships between the various components during the cycle of growth and depletion of the endosperm, some of the results are expressed on a 'per bean' basis.

Q_{O_2} measurements. The oxidative activity of the mitochondrial fractions was measured with α -oxoglutarate (H.M. Chemical Co., Santa Monica, California) as substrate in the presence of the full complement of cofactors (Beever & Walker, 1956) at 25°. A 60 min. oxidation period was allowed and portions were taken at the outset for determination of N.

Analytical. The phospholipid, acid-soluble phosphate ('nucleotide') and RNA contents of the mitochondrial fraction M_2 and the microsomes were determined by the procedure of Ogur & Rosen (1950). The residue after RNA extraction in $HClO_4$ was used for protein determination. In the RNA analyses, the factor 0.35 of Ogur & Rosen was employed to convert the E_{260} (absorption at 260 m μ) readings into RNA phosphorus.

Inorganic phosphate. This was determined by the Fiske & Subbarow (1925) method.

Total nitrogen. Sulphuric acid (5N) was used to digest portions of the various fractions and the residue after RNA extraction, and the NH_3 was determined by nesslerization.

Percentage dry wt. of the particles. After centrifuging, a sample of about 70 mg. fresh wt. was transferred to a small tared bottle and the weight of material determined, before and after drying overnight at 100° (Bartley & Davies, 1954); dry wt. (%) is given by (dry wt./wet wt.) $\times 100$.

RESULTS

By differential centrifuging (see Methods) the crude mitochondrial fraction was separated into two components, M_1 and M_2 . The results in Table 1 show that almost all of the nitrogen of the original crude suspension was recovered in the two sediments, but that the total O_2 uptake of the two fractions was considerably less than the original. Almost all of the oxidative activity which was recovered was concentrated in M_2 , and since this fraction contained about 60% of the original nitrogen, the Q_{O_2} was higher than that of the original crude suspension. The addition of M_1 to M_2 did not repair the loss in total activity, and apparently some irreparable damage attends the separation of the two components. Although the loss in total activity offsets to some extent the gain in specific activity, it is clearly of advantage to use the 'purer' M_2 for comparative chemical analyses; for other purposes the use of the crude mitochondria might be preferred.

The results of analyses of the M_2 fraction are given in Table 2, with values from the original crude suspension for comparison. It will be seen that although about one-half of the acid-soluble phos-

phate and RNA are not found in M_2 , the contents relative to the nitrogen content have increased as a result of the separation.

Changes in the composition of mitochondria and microsomes

Figs. 1 and 2 summarize a series of analyses on the M_2 fraction and the microsomes respectively from the endosperms of seedlings from 3 to 7 days old. The lower graph (B) in each shows the course of the changes in phospholipid and RNA phosphorus and of acid-soluble phosphate relative to the protein-nitrogen content of the particles, while in the upper curves (A) the changes in the absolute levels per individual bean are shown. Up to the fifth day there is a progressive increase in the total amount of mitochondrial protein and subsequently an equally striking decline in this quantity. Accompanying these changes there are parallel changes in phospholipid and RNA. There is, in fact, a relationship between the amounts of RNA and phospholipid on the one hand and the amount of protein nitrogen on the other which is characteristic of the mitochondria of the endosperm regardless of the stage of development of the seedling.

The changes in acid-soluble phosphate, on the contrary, show that at least after the 4 days stage the mitochondria can not be regarded as having a characteristic level of this component. The amount of acid-soluble phosphate increases until the fourth day, but thereafter no extra acid-soluble phosphate accumulates and its concentration, whether expressed 'per bean' or in terms of protein N, shows

Table 1. *Fractionation of mitochondrial suspension*

Fractions M_1 and M_2 were suspended individually in sucrose-phosphate medium so that 1 ml. of this suspension corresponded to 1 ml. of the original suspension; substrate was α -oxoglutaric acid (0.01M). q_{O_2} : $\mu\text{l. of O}_2$ absorbed/mg. of N/hr.

	O_2 uptake $\mu\text{l.}/30$ min.		Total nitrogen mg./ml.		q_{O_2}	
	Expt.		Expt.		Expt.	
	(a)	(b)	(a)	(b)	(a)	(b)
Crude mitochondrial suspension (1 ml.)	116.0	190.0	1.81	1.98	128.0	192.0
M_1 (1000 g/10 min.)	9.3	11.2	0.745	0.725	25.0	30.9
M_2 (20 000 g/20 min.)	66.5	119.0	0.955	1.120	140.0	213.0
$(M_1 + M_2)/\text{crude} \times 100$	65.0	68.8	94.0	93.0	—	—

Table 2. *Chemical analysis of the original crude mitochondrial suspension and the purified mitochondria (M_2)*

(a) and (b) are separate preparations.

		Acid-soluble phosphate		RNA P		Protein N (mg./ml.)
		(μ g./ml.)	(μ g./mg. of protein N)	(μ g./ml.)	(μ g./mg. of protein N)	
Crude mitochondria	(a)	1140	400	39.8	14.0	2.85
	(b)	1180	437	47.3	17.5	2.70
M ₂	(a)	500	650	20.6	26.9	0.77
	(b)	760	670	26.5	23.5	1.13

a sharp decline. We attach significance to this change in attempting to explain the striking decrease in the efficiency of the mitochondria which begins at about the 5 days stage (Akazawa & Beevers, 1957).

The total amount of microsomal protein, in contrast with that of the mitochondria, does not increase during the early stages; a slow decline in protein occurs to the 6 days stage and a sharper fall thereafter. The RNA and phospholipid show parallel changes but the microsomal protein declines more rapidly than these components during the later stages, so that the curves for RNA and phospholipid/mg. of protein nitrogen show a tendency to rise with time. The acid-soluble phosphate is again apparently the first of the various components lost from the microsomes during depletion of the endosperm; the amount relative to the protein nitrogen reaches a very low value after 6-7 days.

Fig. 1. Changes in the composition of the mitochondria (M_2) during the germination of the castor bean. □, Protein N; ●, acid-soluble phosphate; ○, phospholipid P; △, RNA. (A) Results are expressed as amounts of the various components/individual bean endosperm; (B) components are expressed as amounts/unit of protein N.

It will be noted from Figs. 1 and 2 that the absolute levels of the various components are very different in the microsomes and mitochondria. For the purpose of comparing composition more closely, the data of Table 3 may be used. The 4 days stage was used because (a) all the components in M_2 increase together up to this point, and (b) M_2 still shows high oxidative and phosphorylating ability. There is about 50 % more nitrogen in the mitochondria than in the microsomes. The most striking difference is in the specific content of RNA; there is a fivefold superiority in favour of the microsomes. The acid-soluble phosphate content of the M_2 is, on the other hand, twice as great as in the microsomes. The phospholipid content of the mitochondria is roughly two-thirds of that of the microsomes.

Water content of castor-bean mitochondria and its experimental control

Table 4 shows the results of dry-weight determinations on crude mitochondria and on M_1 and M_2 fractions. Under the conditions of isolation the particles from castor beans have a considerably lower water content than those from other tissues

Fig. 2. Changes in the composition of the microsomes during the germination of the castor bean. Other details are as given for Fig. 1.

Table 3. Chemical composition of mitochondria (M_2) and microsomes from 4 days seedlings

	Phospholipid P (μg./mg. of protein N)	Acid-soluble phosphate (μg./mg. of protein N)	RNA P (μg./mg. of protein N)	Protein N (mg./g. of bean)
Mitochondria	36.9	685.0	26.5	0.220
Microsomes	57.0	310.0	134.0	0.148

Table 4. *Dry weight (%) of crude and purified mitochondria*

Age (days)	...	3	4	5	6
Crude mitochondria		30.9	31.1	28.3	25.0
M ₂		—	25.8	25.9	—
M ₁		—	27.0	—	—

Table 5. *Changes in dry weight (%) induced by exposure to media of different tonicities*

In III (a) and (b) refer to separate experiments.

Tonicity of medium in original suspension (M) ...	I	II	III	
	0.02	0.25	0.5	
			(a)	(b)
Treatment		Dry wt. (%) of particles		
None	18.9	24.2	29.5	29.0
0.02 M-Sucrose (30 min.)	17.5	17.3	19.7	—
0.25 M-Sucrose (30 min.)	—	—	—	25.4
0.5 M-Sucrose (30 min.)	28.2	27.6	30.6	30.1
0.8 M-Sucrose (30 min.)	—	—	—	35.0

which have been described (MacFarlane & Spencer, 1953; Bartley & Davies, 1954). After the fourth day there is a progressive increase in the water content.

Changes in the water content can be readily induced by exposing the particles to media of different tonicities, as shown in Table 5, column III. This table also includes data on mitochondria prepared in the usual way except that the washing and final suspension were in media of lower sucrose content (columns I and II). It will be seen that the dry weight of the resulting mitochondria varies with the concentration of the suspending medium. When these mitochondria with different water contents were subsequently exposed to media of different tonicities for 30 min. there were marked changes in weight, which seem to be explicable on simple osmotic considerations. Thus the water content of mitochondria as normally prepared (column III) was much increased by exposure to hypotonic solution (0.02 M) and to a lesser extent by 0.25 M-sucrose. In 0.8 M-sucrose there was a loss of water from the particles. If the mitochondria were allowed to remain in contact with their respective suspending media there was little further change in water content during a 30 min. period; equilibrium had apparently been reached in each instance during the washing, centrifuging and suspension in the cold.

Mitochondria, after treatment with 0.02 M-sucrose ('swollen mitochondria'), lost water on being exposed to 0.5 M-sucrose; apparently the changes in water content are reversible. The mitochondria from 0.25 M-sucrose are particularly interesting because they have initially an intermediate water content, and this can be increased by 0.02 M-sucrose and decreased by 0.5 M-sucrose. None of the following appeared to influence the swelling of

normal mitochondria when they were exposed to media of lower tonicity: temperature (0–30°); 2:4-dinitrophenol (0.002 M); ethylenediaminetetraacetic acid (0.001 M); calcium chloride (0.01 M). In addition, neither the provision of adenosine triphosphate (1 mg./ml.) nor of conditions optimum for oxidative phosphorylation (Akazawa & Beevers, 1957) appeared to affect the swelling and shrinking of washed mitochondrial suspensions.

DISCUSSION

From the data presented here it is clear that the composition of the particles from the endosperm of the 4 days castor bean is not widely different from that of particles of animal origin which have been more fully examined (see, for example, Lindberg & Ernster (1954), Hogeboom & Schneider (1955)). The washed mitochondria have a relatively high level of acid-soluble phosphate and also contain some RNA. The most striking difference in the two kinds of particles examined is in the RNA content; the microsomes have roughly five times as much RNA phosphorus/mg. of protein nitrogen as the mitochondria. Although we have no data at present which would allow us to ascribe the presence of RNA in the mitochondria to a small microsomal contamination, this must remain a possibility.

In so far as our results can be compared to those of Kmetec & Newcomb (1956) it would appear that the RNA content of the castor-bean microsomes is considerably higher than those from peanut. One important difference in the two preparations should be noted, however: the peanut particles were obtained at much lower centrifugal forces, and thus the separation of the microsomal particles from the castor bean may have been more effective. Although the relative RNA contents of the 'mitochondrial'

and 'microsomal' fractions reported by Stafford (1951) are closer to those now described for castor-bean particles, the differences in preparative procedure should be noted here also.

Martin & Morton (1956*a*) provide data and rigid tests for the reliability of the procedures employed, which show that in silver-beet petiole the RNA content of the microsomes is not nearly so widely different from that of the mitochondria as it is in the particles of animal origin, and draw attention to this difference. They point out, however, that the metabolic status of the tissue may be an important consideration, and they have provided data from germinating-wheat roots in which the RNA content of the microsomes is considerably higher (Martin & Morton, 1956*b*). In the castor-bean endosperm, a highly active material, the RNA level in the microsomes is not only five times as great as that in the mitochondria, but the absolute levels observed (on a protein-nitrogen basis) are close to those observed by Palade & Siekewitz (1956) for liver microsomes.

The day-to-day changes in the composition of the mitochondria have been compared with those occurring in the microsomes. No allowance has been made in this or in the succeeding paper (Akazawa & Beevers, 1957) for possible differences in the degree of completion with which the particles can be extracted and recovered from tissues of different ages by the standard procedure employed. Particular attention has been drawn to the striking changes in the acid-soluble phosphate content of the mitochondria, changes which do not follow those of the other components examined. The content of 6 days beans is only one-sixth of that at 4-5 days. In the microsomes also this component is the first to decline sharply.

The water content of the mitochondria is relatively low at all stages of development and regardless of the extent of swelling. Values of between 19 and 35 % dry weight are to be compared with those of sheep-kidney mitochondria [8-20 % dry weight (Bartley & Davies, 1954)] and of rat liver [11-22 % dry weight (MacFarlane & Spencer, 1953)]. Experiments with mitochondria from 4 days beans have shown that exposure to media of different tonicities results in rapid changes in the water content, pointing to a further similarity between mitochondria from animals and plants; a similarity which had in fact been inferred earlier from other kinds of experiments (Laties, 1953; and Farrant *et al.* 1953).

SUMMARY

1. Castor-bean mitochondria and microsomes prepared by differential centrifuging of homo-

genates in sucrose-phosphate have been analysed for ribonucleic acid (RNA), acid-soluble phosphate, phospholipid and protein.

2. Expressed on a protein basis the mitochondria have relatively low phospholipid and RNA contents, whereas the acid-soluble phosphate is higher than that of the microsomes. These facts, and particularly the very high RNA content of the microsomes, point to a general similarity to corresponding particles of animal origin.

3. During the first 4 days of germination there is an increase in mitochondrial protein and this is accompanied by an increase in all of the other components. After the fifth day these components decrease, especially the acid-soluble phosphate. The proportion of RNA and phospholipid to protein nitrogen remains more or less constant throughout, but that for acid-soluble phosphate declines.

4. A similar decline in acid-soluble phosphate is noted in the microsomes; in these particles there is no increase in protein or other components in the first few days of germination.

5. The water content of the mitochondria has been determined and its variation in response to media of different tonicity has been measured.

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