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Mitochondrial Preparations from the Leaves of Tobacco (*Nicotiana tabacum*)

4. SEPARATION OF SOME COMPONENTS BY DENSITY-GRADIENT CENTRIFUGING*

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Extracts of animal tissues have frequently been fractionated by centrifuging in stabilized density gradients. Sometimes the process is used to fractionate the extracts into nuclear, mitochondrial and microsomal fractions (Anderson, 1956), but more often to examine, in greater detail, mitochondrial and microsomal preparations made by differential centrifuging (de Duve *et al.* 1960). The process has occasionally been applied to plant-tissue extracts, as when James & Das (1957) used a simplified system to separate chloroplasts from leaf extracts. However, the full resolving power of the technique, as illustrated by the experiments of de Duve *et al.* (1960), has not been exploited with plant extracts.

This paper describes a sucrose-gradient procedure, which uses only low centrifugal forces, and its application to the chloroplast and mitochondrial fractions prepared from tobacco leaves (Pierpoint, 1959). It partially separates succinoxidase activity from the chloroplast-bearing particles, and enables the distribution of fumarase, cytochrome oxidase and glycolic oxidase among the different particles to be measured.

EXPERIMENTAL

Chloroplast preparation. Tobacco leaves (*Nicotiana tabacum*, White Burley strain) (25–40 g.) were disrupted in five to six times their own weight of medium (sucrose,

0.4M; tris-HCl buffer, 0.2M; K_2HPO_4 , 0.01M; EDTA, 5 mM; trisodium citrate, 0.02M; final pH 7.7), by using the method described by Pierpoint (1959). The extract was passed through two layers of cheese-cloth, and centrifuged for 1 min. at a centrifugal force of approx. 500g to remove sand, fibre residue and starch grains. A second centrifuging for 7 min. at 1000g gave a dark-green precipitate rich in intact chloroplasts, which was usually washed by suspending it in the washing medium (Pierpoint, 1959) and resedimenting it at 1000g. It was then evenly suspended in 15 ml. of a solution containing sucrose (0.15M), tris-HCl buffer, pH 7.4 (5 mM), and EDTA (1 mM).

When mitochondrial and chloroplast preparations were made from the same batch of leaves, liquid in which the chloroplasts had been washed was added to the supernatant fluid from which the mitochondria were to be sedimented.

Mitochondrial preparations. Mitochondrial preparations were made from tobacco leaves (25–40 g.) by the method described (Pierpoint, 1959, 1960a). They were not washed, but suspended in 10–15 ml. of a solution containing sucrose (0.15M), tris-HCl buffer, pH 7.4 (5 mM), and EDTA (1 mM).

Centrifuging in sucrose gradients. Sucrose gradients were made by layering sucrose solutions of decreasing densities in the 100 ml. glass tubes of an MSE Major centrifuge. The solutions of sucrose generally used were 1.76M (20 ml.), 1.4M (6 ml.), 1.1M (10 ml.), 0.8M (6 ml.), 0.5M (6 ml.) and 0.3M (10 ml.); all except the most concentrated one was buffered at pH 7.4 with tris buffer (0.01M). The chloroplast or mitochondrial preparations (10 ml.) were pipetted on the top after the interfaces of the layers had been allowed to smooth by standing for at least 5 hr. in the cold (0–5°). The tubes were then fitted with plastic caps and centrifuged in

* Part 3: Pierpoint (1960b).

the cold at approx. 1000g for 30 min. The centrifuge was accelerated and decelerated slowly to minimize swirling of the tube contents.

After centrifuging, the contents of the centrifuge tubes were fractionated with a sliding-section device similar to that described by Anderson (1956). The centrifuge tube was held against the lower of two closely fitting Perspex blocks and sealed in place with a little lanolin. A narrow stainless-steel tube penetrated these blocks and passed to the bottom of the centrifuge tube. As a dense sucrose (1.76M) solution flowed through this steel tube to the bottom of the centrifuge tube, the layers of sucrose were lifted out of the tube into the corresponding holes in the two Perspex blocks. The layer of solution (6–7 ml.) which had entered the hole in the top block was isolated by sliding this block over the lower one, and was then removed with a pipette. Fractionation of the sucrose solution was continued until the last fraction was an optically clear sample of the dense (1.76M) sucrose.

Enzyme estimations. Succinoxidase was measured manometrically as described by Pierpoint (1959). Samples (1.4 ml.) of the fractions taken from the sucrose gradients were incubated in a medium of composition: sodium succinate, 40 μ moles; sucrose, 0.6 M-mole; phosphate buffer, pH 7.3, 0.1 M-mole; magnesium sulphate, 10 μ moles; cytochrome *c*, 0.018 μ mole; adenosine triphosphate, 2 μ moles; diphosphopyridine nucleotide, 0.2 μ mole; the final volume was 2.5 ml., and the pH 7.2–7.3. Oxygen uptake (μ l.) was followed for 1 hr.

The final concentration of sucrose in the manometer flasks depended on the fraction of the sucrose gradient which was being tested; however, the sucrose in the incubation medium was always enough to raise the concentration in all flasks into the region (0.32–1.00M) where succinoxidase activity varied only slightly (<10%) with sucrose concentration.

Glycolic oxidase and fumarase were measured as described (Pierpoint, 1960b), and activity was expressed as μ l. of O₂ uptake/ml./30 min. and $\Delta E_{240\text{ m}\mu}/0.2\text{ ml.}/15\text{ min.}$ respectively. Both activities were slightly decreased (about 10%) by differences in the sucrose content of the solutions tested.

Cytochrome oxidase was measured spectrophotometrically as described by Simon (1958). The spectrophotometer cell contained sucrose (0.6 M-mole), phosphate buffer (0.30 M-mole; pH 7.4), reduced cytochrome *c* (70 μ M-moles) and usually 0.04 ml. of enzyme solution. The extinction at 550 m μ was followed in a Unicam SP. 500 spectrophotometer at 1 min. intervals for 5 min., and again after adding 0.02 ml. of $\text{M-K}_3\text{Fe}(\text{CN})_6$. The reaction followed first-order kinetics, and the rate constant (*k*) for 0.04 ml. of enzyme was calculated according to Smith & Conrad (1956). Multiplying *k* by the concentration of reduced cytochrome *c* (24 μ M) gave the rate of oxidation of cytochrome *c* (mole/l./min.).

The oxidation measured in this manner was directly proportional to the amount of added enzyme. Activity was decreased by higher concentrations of cytochrome *c*, as is that of horse-heart-muscle enzyme (Smith & Conrad, 1956), and it was affected by alterations in both pH and phosphate concentration. The phosphate buffer used held both these quantities at near-optimum levels.

In some experiments cytochrome oxidase was activated by digitonin, by using the procedure described by Simon

(1958). A sample of the enzyme solution (0.5 ml.) was pipetted into a tube containing 10 mg. of digitonin and, after stirring for 30 sec., a sample of the solution was taken for immediate estimation.

Chemical estimations. Protein N was estimated by a micro-Kjeldahl method (Pierpoint, 1959), and chlorophyll by the method of Arnon (1949).

Recovery of material from sucrose gradients. Fumarase (80–85%) and over 90% of the cytochrome oxidase added to the sucrose gradients could be recovered in the separated fractions. The recovery of glycolic oxidase was about 60%; the loss could not be accounted for entirely by the effect of sucrose on the enzyme estimation. Although the reason for this loss of activity is not definitely known, experiments have shown that the stability of glycolic oxidase at 30° is decreased appreciably by sucrose and tris-HCl buffer.

Chlorophyll recovery was usually 85–95%, but in one experiment (Table 2) it was apparently 60%. This low recovery was probably due to an error in the chlorophyll estimation, as the recovery of fumarase in the same experiment was quite satisfactory (80%). The recovery of protein N, measured in one experiment (Table 1), was 93%.

RESULTS

Distribution of chlorophyll and protein nitrogen in sucrose gradients

The bulk (70–90%) of the chlorophyll in the mitochondrial preparations was dispersed in the top 20 ml. of the layered sucrose solutions after being centrifuged at 1000g for 30 min. Its position was not altered appreciably when the centrifuging was continued for 60 min. When the tube contents were divided into 6–7 ml. fractions, the second one invariably had the highest chlorophyll content (Table 1); on average it was 15% higher than the

Table 1. *Distribution of chlorophyll and protein nitrogen in sucrose gradients*

A mitochondrial preparation (10 ml.) was layered on a sucrose gradient and centrifuged as described in the Experimental section. The contents of the tube were fractionated, and chlorophyll and protein N determined on samples of each fraction. The density values were measured on similar fractions taken from a sucrose gradient that had been 'smoothed' and centrifuged, but to which no plant extract had been added.

Fraction no.	Density (g./ml.)	Protein N (μ g./ml.)	Chlorophyll (μ g./ml.)
1	1.017	370	230
2	1.021	385	300
3	1.036	200	150
4	1.045	105	50
5	1.074	55	30
6	1.127	45	15
7	1.141	25	5
8	1.162	20	5
9	1.199	—	—
Initial mitochondrial preparation	—	840	580

Table 2. *Distribution of succinoxidase, fumarase and chlorophyll after centrifuging in sucrose gradients*

Mitochondrial and chloroplast preparations were made from the same batch of tobacco leaves, and centrifuged for 35 min. at 1000g in sucrose gradients. The contents of the centrifuge tubes were divided into ten fractions, the top ones being labelled 1 and the bottom ones 10, and succinoxidase, fumarase and chlorophyll determined in samples of each.

Fraction no.	Succinoxidase ($\mu\text{l./1.4 ml./hr.}$)		Fumarase ($\Delta E_{240 m\mu}/0.2 \text{ ml./15 min.}$)		Chlorophyll ($\mu\text{g./ml.}$)	
	Mitochondria	Chloroplasts	Mitochondria	Chloroplasts	Mitochondria	Chloroplasts
1	10	3	0.06	0.008	160	22
2	24	1.7	0.12	0.008	180	25
3	63	6.5	0.17	0.008	130	14
4	62	7.5	0.20	0.012	59	10
5	42	9.5	0.12	0	36	10
6	8	0	0.05	0	29	40
7	4	2	0.03	0	23	100
8	0	0	0.02	0	23	300
9	0	0	0	0	16	140
10	0	0	0	0	5	14

first. No intact chloroplasts were revealed by microscopic examination of the top three fractions.

In contrast, most (90 %) of the chlorophyll of washed chloroplast preparations was dispersed in the 20 ml. of solution (constituting fractions nos. 7-9) immediately above the clear layer of 1.76M-sucrose (Table 2), and was associated with many intact chloroplasts. Only a little chlorophyll and no detectable chloroplasts were left in the top layer of sucrose. Sometimes a trace of chlorophyll was attached to cell debris and descended, along with some starch grains, through the 1.76M-sucrose to the bottom of the tube.

The protein nitrogen of mitochondrial preparations sediments through the sucrose gradients in a manner similar to the chlorophyll (Table 1). This is expected when three-quarters of the protein in a preparation is derived from chloroplasts (Pierpoint, 1959). The ratio chlorophyll: protein nitrogen is lowest in fractions collected from the lower part of the sucrose gradient, suggesting a larger proportion of non-chloroplast protein in these fractions.

Distribution of enzymes in the sucrose gradients

Succinoxidase. Most (90-95 %) of the succinoxidase of a tobacco-leaf extract is associated with the mitochondrial preparation and the remainder with the chloroplast preparation (Pierpoint, 1959). When the two preparations were centrifuged in sucrose gradients, their succinoxidase activity sedimented to the same level (Table 2). It was recovered mainly in the third, fourth and fifth fractions, showing a distribution unlike that of chloroplasts and their fragments. The succinoxidase activity ($\mu\text{l.}$ of oxygen uptake/mg. of protein N/30 min.) of these fractions was 2.5-3 times greater than that of the mitochondrial preparations from which they were prepared, owing to the separation of chloroplast protein.

Fumarase. This was distributed among fractions of sucrose gradients in a similar way to succinoxidase (Table 2). The activity, 95 % of which was in the mitochondrial preparations, was highest in the fourth fractions. Although the succinoxidase and fumarase activities of the different fractions of Table 2 varied over a 30-40-fold range, the ratio of the two activities in each fraction varied only 2-3-fold.

Glycolic oxidase. Very little of the glycolic oxidase of tobacco-leaf extracts was sedimented at centrifugal forces up to 55 000g (Pierpoint, 1959), but a few per cent were present in mitochondrial and chloroplast preparations even after washing. Table 3 shows that the enzyme in both types of preparation remained in the top half of the tube after centrifuging in a sucrose gradient. Its distribution most resembled that of chloroplast fragments, but in three experiments the concentration was always higher (15-50 %) in the first fraction than the second. No activity could be detected in fractions containing intact chloroplasts, not even after disruptive treatments such as repeated freezing and thawing, or exposure to digitonin (15 mg./ml.).

Cytochrome oxidase. The cytochrome oxidase of tobacco-leaf extracts, like that of other plant tissues (Hackett, 1959), is readily sedimented. After fractionation of the extracts, 70-90 % of the activity that can be recovered is in the mitochondrial preparation, and the rest is fairly evenly distributed between the chloroplast preparation and the unsedimented residue. The proportion of the total activity of the leaf extract that is recovered in the three fractions is very high (160-290 %) when the cytochrome-oxidase estimations are performed immediately after the preparations are made. It is lower (80-110 %) if the preparations are stored on ice for 12-24 hr. before the estimations. This decrease in recovery is due not to any

appreciable alteration in the activity in the chloroplast and mitochondrial fractions, but to an increase in the activity detectable in the initial extract (Table 4). The low activity in the supernatant fraction also increases during this storage.

It is not known if this increase is due to the destruction of an inhibitor present in these two fractions or to alterations in the state of the cytochrome-oxidase-bearing particles that increases the accessibility of the enzyme to added substrate.

Table 3. *Distribution of fumarase, glycollic oxidase and chlorophyll after centrifuging in sucrose gradients*

The chloroplasts and mitochondrial preparations were made from different batches of tobacco leaves, layered on sucrose gradients and centrifuged, and then the tube contents fractionated. The first fractions were taken from the tops of the tubes, and the ninth from near the bottoms.

Fraction no.	Fumarase ($\Delta E_{240\text{ m}\mu}/0.2\text{ ml./15 min.}$)		Glycollic oxidase ($\mu\text{l./30 min./ml.}$)		Chlorophyll ($\mu\text{g./ml.}$)	
	Mitochondria	Chloroplasts	Mitochondria	Chloroplasts	Mitochondria	Chloroplasts
1	0.04	—	209	18.5	165	54
2	0.08	—	142	7.6	210	58
3	0.12	—	39	3.5	125	36
4	0.22	—	30	3	58	22
5	0.14	—	19	2	36	36
6	0.05	—	0	0	25	155
7	0.02	—	0	0	19	310
8	0.006	—	0	0	16	115
9	0	—	0	0	0	18

Table 4. *Effect of storage and digitonin on the cytochrome oxidase of tobacco-leaf extracts*

An extract of tobacco leaves (25 g. in 150 ml. of extraction medium) was fractionated by centrifuging, and the mitochondrial and chloroplast preparations were washed before being suspended in a solution containing sucrose (0.15M), tris-HCl buffer, pH 7.4 (5 mM), and EDTA (1 mM). Cytochrome oxidase was estimated immediately after fractionation was completed and again after the preparations had been stored on ice for 24 hr. The observed rates of oxidation of cytochrome *c* were multiplied by the ratio total volume of fraction : volume of sample in estimation, to give the total enzyme activity (m-moles/l./min.) of each fraction.

	Protein N (mg.)	Cytochrome oxidase of fresh preparations		Cytochrome oxidase of stored preparations	
		Total activity	Total activity after digitonin treatment	Total activity	Total activity after digitonin treatment
Initial leaf extract	73	0.98	9.60	2.82	7.00
Chloroplast fraction	14.6	0.21	0.78	0.28	0.67
Mitochondrial fraction	9.8	2.5	8.30	2.60	7.40
Supernatant fraction	49	0.11	1.34	0.24	0.90
Percentage of initial activity recovered in the 3 fractions	—	287	109	110	127

Table 5. *Distribution of succinoxidase, cytochrome oxidase and chlorophyll in sucrose gradients*

Mitochondrial and chloroplast preparations were made from separate batches of tobacco leaves and centrifuged in sucrose gradients. The centrifuge-tube contents were divided into nine fractions (the ninth being taken from near the bottom of the tubes) and succinoxidase, cytochrome oxidase and chlorophyll estimated in the fractions.

Fraction no.	Succinoxidase ($\mu\text{l./1.4 ml./hr.}$)		Cytochrome oxidase ($\mu\text{moles/l./min.}$)		Chlorophyll ($\mu\text{g./ml.}$)	
	Mitochondria	Chloroplasts	Mitochondria	Chloroplasts	Mitochondria	Chloroplasts
1	7	—	2.1	0.12	290	62
2	17	—	3.6	0.19	370	72
3	37	—	3.74	0.16	210	50
4	40	—	1.84	0.06	83	36
5	20	—	0.53	0	51	50
6	5	—	0.24	0	29	152
7	3	—	0.16	0.04	14	620
8	0	—	0	0	11	200
9	0	—	0	0	0	0

A brief exposure to digitonin (20 mg./ml.) increases the cytochrome oxidase activity of many plant tissues, probably by disorganizing lipoprotein membranes (Simon, 1958). This treatment also increased the activity of both fresh and aged tobacco-leaf preparations (Table 4). The increase was greatest (10–12-fold) in the fresh unfractionated leaf extracts and the supernatant fraction. The factor that is responsible for the initial low activity of these two extracts is apparently not active after the digitonin treatment.

When the mitochondrial and chloroplast preparations were centrifuged in the sucrose gradient, the cytochrome oxidase behaved similarly to, but not identically with, succinoxidase (Table 5). Cytochrome oxidase sedimented less completely than the other, so that the ratio of cytochrome oxidase to succinoxidase was highest in the first fraction and only one-tenth as much in the fourth and fifth fractions. Treatment with digitonin increased three- to five-fold the cytochrome oxidase of a number of these fractions. Although an increase also occurred in the activity of chloroplasts purified in the sucrose gradient, the activity was still too small to be measured accurately.

DISCUSSION

A little more than three-quarters of the protein contained in a mitochondrial preparation from tobacco leaves is derived from chloroplasts (Table 1; and Pierpoint, 1959). This is not completely separated from the other protein by the sucrose-gradient centrifuging, but is separated sufficiently to provide direct evidence on the enzymic properties of the two fractions. Fumarase and succinoxidase behave as if they were attached to a class of particles which sediments further than the chloroplast fragments, but not so far as intact chloroplasts. These particles are probably the mitochondria-like bodies whose existence in these preparations has been seen in the electron micrographs previously mentioned (Pierpoint, 1960*a*).

Most of the cytochrome oxidase in the preparations also seems to belong to this type of particle, although some of it does not sediment so far as the succinoxidase. This less-sedimented portion is unlikely to be attached to chloroplast fragments, as no activity could be detected in intact chloroplasts before or after fragmentation with digitonin. It is probably attached to damaged or fragmented mitochondria that have lost their ability to oxidize succinic acid. Liver mitochondria have been disintegrated to produce similar particles that have cytochrome oxidase but no succinoxidase activity (Crane & Glenn, 1957).

Jagendorf & Wildman (1954) and McClendon (1953) found no parallel between the chlorophyll

and cytochrome oxidase in fractions of tobacco-leaf extracts, and they also concluded that the enzyme was located on the mitochondria. However, similar experiments which gave different results led Sissakian & Filippovich (1956) to suggest that the enzyme is attached to the chloroplasts. Sissakian (1958) attributes these differences in results to the formation of inhibitory polyphenols from quinol in the manometric cytochrome-oxidase estimations of the other workers. This criticism, however, does not apply to the sensitive spectrophotometric method of enzyme assay used in the present work, as it does not require quinol. A more likely reason for these differences in results is the small volume of buffered sucrose used by Sissakian & Filippovich (1956) for grinding tobacco leaves (150 ml. of liquid/250 g. of leaf). Their leaf extracts were about ten times more concentrated than those of other workers. The higher salt concentrations and lower pH values of these extracts may facilitate the agglutination of the mitochondria and render them sedimentable at the low centrifugal forces that sediment chloroplasts. Animal mitochondria are sedimented by centrifuging at 2000*g* for 5–10 min. after standing in 1.5 mM-potassium chloride (Kennedy & Lehninger, 1949).

Pierpoint (1960*b*) gave evidence for thinking that glycolic oxidase is a 'soluble' enzyme, unassociated with either mitochondria or chloroplasts. Since then Zelitch & Barber (1960) have emphasized that some glycolic oxidase remains in spinach chloroplasts even after four washings. Tobacco chloroplasts also contain some activity after two washings, as do tobacco mitochondrial preparations. But, as Table 3 shows, the enzyme in both these preparations does not behave, on being centrifuged in a sucrose gradient, as if it were attached to either chloroplasts or mitochondria. Nor does it behave exactly as if it were attached to chloroplast fragments, a conclusion supported by the absence of activity in intact chloroplasts and chloroplasts disrupted by freezing or digitonin. It is unlikely that the concentrated sucrose solutions of the gradient have brought about a dissociation of the enzyme from these particles, as chloroplast and mitochondrial preparations washed in media containing 0.8–1.1*M*-sucrose have no less glycolic oxidase than preparations washed in the normal medium containing 0.2*M*-sucrose. The most probable explanation of the behaviour of glycolic oxidase in the gradients is that it is a 'soluble' enzyme which is spread beyond the top layers of the sucrose by some mixing action, caused perhaps by the sedimentation of the particles.

It may become necessary to revise this conclusion when more is known of the enzymic

activities of chloroplasts isolated in non-aqueous media (Stocking, 1959), and which have not been leached of some water-soluble components. But the suggested role of glycollic oxidase in the respiration of illuminated leaves (Zelitch, 1959) does not necessarily conflict with glycollic oxidase occurring outside chloroplasts; although glycollic acid is formed in chloroplasts, unlike some other products of photosynthesis it diffuses readily into the surrounding cytoplasm (Tolbert, 1959).

SUMMARY

1. Preparations from tobacco leaves were centrifuged in sucrose density gradients and resolved into chloroplasts, chloroplast fragments and particles, presumably mitochondria, containing succinoxidase activity.

2. The centrifuging sedimented fumarase as if it were attached to the mitochondria.

3. Cytochrome oxidase sedimented as if it were associated with mitochondria, and some mitochondrial fragments. No activity was detected in chloroplasts either before or after disruption with digitonin.

4. Glycollic oxidase did not sediment appreciably during the centrifuging. It could not be detected in intact chloroplasts.

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The Effect of Sulphate Assimilation on the Induction of Arylsulphatase Synthesis in Fungi

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Arylsulphatases of various types are present in most animals, including mammals, birds, amphibia and molluscs (Dodgson & Spencer, 1956). In bacteria the enzyme is not widely distributed but it has been found in strains of *Aerobacter aerogenes* (Harada, 1952), *Salmonella*, mycobacteria (Whitehead, Morrison & Young, 1952), *Streptococcus alcalophilus* (Harada, Kono & Yagi, 1954), *Alcaligenes metalcaligenes* (Dodgson, Melville, Spencer & Williams, 1954) and in other isolated species. In fungi, the arylsulphatase of Taka-diestase (Neuberg & Korono, 1923) and other commercial prepara-

tions (Abbot, 1947) of *Aspergillus oryzae* has long been known and the presence of the enzyme in other fungi has been noted (Harada *et al.* 1954).

The only sulphate ester known to be present in fungi, apart from the sulphatophosphates, adenosine 5'-sulphatophosphate and adenosine 3'-phosphate 5'-sulphatophosphate (Spencer & Harada, 1960), is choline sulphate which appears to be restricted to higher fungi (Harada & Spencer, 1960). It was possible that choline sulphate or the sulphate-phosphate anhydrides could act as endogenous inducers of arylsulphatase synthesis and to