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The Effect of Digitonin on the Cytochrome *c* Oxidase Activity of Plant Mitochondria

By E. W. SIMON

Department of Botany, The Queen's University, Belfast

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The activity of an enzyme is given by the rate at which it causes its substrates to react under defined conditions. To determine this activity it is necessary to create an experimental situation in which the substrates have free access to the enzyme, since any barrier between enzyme and substrates will appear to reduce the activity of the enzyme.

Cytochrome *c* oxidase promotes the aerobic oxidation of cytochrome *c*, a substrate of relatively large molecular dimensions with a molecular weight of about 12 000 (Wainio & Cooperstein, 1956). It is hardly to be expected that so large a molecule will penetrate freely into cells—indeed experiment has shown that exogenous cytochrome *c* does not penetrate into rat-tissue cells (Beinert & Reissmann, 1949)—and it is therefore necessary to break cells to measure the activity of their cytochrome oxidase. If the cells are ground in the appropriate buffered sucrose medium, centrifuging the resulting homogenate will yield mitochondria which bear cytochrome oxidase in addition to many other enzymes. Cytochrome oxidase-bearing particles can also be isolated from homogenates made by grinding cells in plain buffer solutions (Keilin & Hartree, 1947; Bhagvat & Hill, 1951). These particles, which are fragments of the original mitochondria, lack many of the activities of the intact mitochondria but they possess nevertheless several other enzymes besides cytochrome oxidase (Keilin & Hartree, 1947). The test system for measuring cytochrome *c* oxidase activity therefore consists of a substrate (cytochrome *c*) of large molecular size and a particulate enzyme system in which the oxidase is bound to other enzymes. It seems reasonable to suppose that the cytochrome *c* supplied exogenously can react only with oxidase molecules located at the surface of the particles

(Cleland & Slater, 1953), any oxidase molecules lying within the particles being inaccessible to the substrate. In support of this postulate there is evidence that procedures which disrupt the particles yield preparations having a higher cytochrome oxidase activity. Thus Wainio & Aronoff (1955) found that a variety of surface-active agents increased the cytochrome oxidase activity of a Keilin and Hartree heart-muscle preparation. Mackler & Green (1956) obtained increases in a heart preparation with deoxycholate and Simon (1957) described experiments in which the cytochrome oxidase activity of *Arum* mitochondria was increased after mechanical vibration or treatment with digitonin.

Little attempt has been made so far to determine how much cytochrome *c* oxidase activity can be revealed by such procedures, although this information may have an important bearing on our understanding of oxidation processes in those tissues which appear at the moment to be deficient or lacking in cytochrome oxidase. The action of digitonin was selected for further study as the work of Wainio & Aronoff (1955) and Simon (1957) suggested that it was particularly active. The present paper describes experiments on the action of digitonin in which particles isolated from bean shoots were used as a source of cytochrome oxidase. It is shown that incubation of the particles with digitonin for a short time increases cytochrome oxidase activity by a substantial amount.

MATERIALS AND METHODS

Plant material. Seeds of *Phaseolus vulgaris* (French bean) were sown in moist sand in covered glass containers and incubated for 5 days at 25° in the dark with occasional weak

red light. The plumular hooks and roots were excised, leaving the white hypocotyls (about 2 in. long). Seeds of *Ricinus* (castor bean) were grown under the same conditions. Fruits and vegetables were bought locally; leek roots were cut off plants grown in garden soil.

Preparation of mitochondria. Plant material (30 g.) was ground in a pestle and mortar with a little quartz sand and 45 ml. of a medium containing 0.2M-sucrose and 0.05M Sørensen phosphate buffer, pH 7.1. After straining through muslin the mixture was centrifuged at 500 g for 10 min. and the resulting supernatant then centrifuged for 15 min. at 4000 g. The mitochondrial pellet was resuspended in the above medium with a glass homogenizer, the mitochondria sedimented again by centrifuging for 15 min. at 4000 g and finally suspended in 3.25 ml. of the medium. For the experiments presented in Table 4 this procedure was modified: the centrifugings were made at 1000 g for 5 min. and 10 000 g for 15 min. respectively and the final preparations contained the mitochondria from about 15 g. of plant material in a volume of 3.25 ml. The preparations were made at 2° and when the final suspension of mitochondria had to be stored between assays it was placed in a mixture of ice and water.

Chemical materials. Catalase and trypsin (manufacturer's designation 1-300) were purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.), dodecyl sodium sulphate from the Watford Chemical Co. Ltd. (London E. 3) and A.R. digitonin from Hopkin and Williams Ltd. Air-dried venom of *Crotalus atrox* was supplied by the Zoological Society of London. Cytochrome *c* was prepared from ox heart according to Keilin & Hartree (1945).

Assay of cytochrome *c* oxidase

Spectrophotometric procedure. A volume (0.01 ml.) of the enzyme preparation was pipetted into a spectrophotometer cell of 1 cm. light-path loaded with 3 ml. of a medium containing sucrose (0.2M), phosphate (0.05M, pH 7.1) and cytochrome *c* (20 μ M), the cytochrome *c* being reduced with dithionite and then oxygenated to remove the excess. Readings of optical density at 550 m μ were made with a Unicam SP. 500 spectrophotometer at intervals of 15 sec. for 90 sec. and then again after the addition of 0.02 ml. of M-potassium ferricyanide to oxidize the cytochrome *c*. The first-order rate constant for the disappearance of reduced cytochrome *c* was calculated according to Smith (1955). Suspensions of mitochondria not treated with digitonin usually had a low activity, and it was sometimes found that a minute or two passed before the attainment of the maximum rate of change of optical density. Readings were then continued for 3 min. and the highest rate/min. recorded in this period was used to calculate the rate constant. The rate constant (*k*) for 0.01 ml. of enzyme preparation and expressed in min.⁻¹ was multiplied by the concentration of reduced cytochrome *c* (20 μ M) to yield the rate of oxidation of cytochrome *c* ($20 \times 10^{-6} \times k$ mole. l.⁻¹ min.⁻¹). Since four molecules of reduced cytochrome *c* are required to oxidize one molecule of oxygen to water, the rate of oxygen uptake was $5 \times 10^{-6} \times k$ mole. l.⁻¹ min.⁻¹. From this it can be calculated that the rate of oxygen uptake by 0.5 ml. of enzyme preparation in a reaction volume of 3 ml. is $10^3 \times k$ μ l./hr., and this figure was used to determine the rate of oxygen uptake as μ l. of O₂/hr./g. wet wt. of tissue.

Manometric procedure. This followed the method described by Simon (1957).

RESULTS

Action of digitonin on mitochondrial cytochrome oxidase

The action of digitonin is dependent on its concentration and on the time for which it acts on a suspension of mitochondria. It is clear from Fig. 1 that cytochrome oxidase activity is greater in the presence of digitonin than in its absence but that activity decreases if the digitonin is held in contact with the mitochondria for long. In all subsequent experiments a sample was removed for assay 30 sec. after the addition of digitonin to the mitochondria. The effect of digitonin depends on its concentration if this is lower than about 10 mg./ml. (see Fig. 2). For subsequent work, a concentration of 20 mg./ml. was chosen. The digitonin becomes diluted 300 times in the assay procedure, 0.01 ml. of suspension being pipetted into 3 ml. of cytochrome *c* solution, and it is then too weak to have any further action on the mitochondria.

A series of unexpected results led to the discovery that storing a suspension of mitochondria at 0° for a period decreases the cytochrome oxidase activity, which can then be elicited by treatment with digitonin. In Fig. 3 are shown the results of an experiment in which a preparation of mitochondria was held at 0° and samples were removed at 12 min. intervals for incubation with digitonin and assay. The activity of control samples, on the other hand, remains constant for long periods (Fig. 1, curve A), and the procedure was therefore

Fig. 1. Cytochrome oxidase activity in *Phaseolus* mitochondria after incubation for varying times with (A) 0, (B) 0.5, (C) 2 and (D) 20 mg. of digitonin/ml. The first readings in A, B and C were taken after incubation for 30 sec. with digitonin; the results are from three separate experiments.

adopted of working rapidly, treating preparations with digitonin as soon as possible and doing the control experiment after the digitonin treatment.

The oxidation of cytochrome *c* by digitonin-treated mitochondria followed a first-order law. The first-order rate constant varied with cytochrome *c* concentration, the value with $20\mu\text{M}$ -cytochrome *c* being about half that with $8\mu\text{M}$. Although this variation could be studied only over a relatively narrow range of cytochrome concentrations the results confirm the findings of Smith & Conrad (1956). According to these authors cytochrome *c* acts as an inhibitor, decreasing the rate constant for the oxidation when it is present in high concentrations, from which it follows that Slater's (1949) extrapolation method of determining cytochrome oxidase activity at an infinite concentration must underestimate the full activity of the oxidase. It was decided to confine subsequent measurements to one concentration of cytochrome *c*, namely $20\mu\text{M}$.

The oxidation of cytochrome *c* by mitochondria treated with digitonin proceeds to completion, and if the cytochrome *c* is then reduced by the addition of a small amount of dithionite the process of oxidation is repeated. Digitonin *per se* does not oxidize reduced cytochrome *c*. The addition of catalase (in quantity sufficient to halve the optical density of a solution of hydrogen peroxide at $240\text{ m}\mu$ in

75 sec.) did not affect the rate of oxidation of cytochrome *c* by a mitochondrial suspension; thus a cytochrome *c* peroxidase is unlikely to be acting.

Assay procedure. The mitochondrial suspension was prepared as described above. Within 1 min. of the completion of the preparation 0.5 ml. of the suspension was pipetted into a cold tube containing 10 mg. of digitonin. The digitonin was brought into solution by rubbing it against the sides of the tube with a glass rod. After 30 sec. 0.01 ml. of the

Fig. 2. Effect of incubation for 30 sec. with varying concentrations of digitonin on cytochrome oxidase activity in *Phaseolus* mitochondria. The several concentrations were tested in serial order at 3 min. intervals, starting with the strongest.

Fig. 3. Effect of incubating a mitochondrial suspension at 0° for various times before treating with digitonin (20 mg./ml. for 30 sec.) and then assaying with either $20\mu\text{M}$ - or $10\mu\text{M}$ -reduced cytochrome *c*.

Fig. 4. Relationship between the volume of digitonin-treated mitochondrial suspension assayed and the activity determined by the standard procedure. Assays were made at 3 min. intervals and in the order indicated.

mixture was assayed. The activity of the untreated mitochondrial suspension was assayed next, about 4 min. after the preparation of the mitochondria. Experiment showed that reduction of the period of incubation with digitonin from 30 sec. to 15 or 10 sec. increased activity by 20 or 30 %, but it was found difficult to carry through the necessary manipulations in a reproducible manner in periods much shorter than 30 sec. The cytochrome oxidase activity recorded in this procedure is proportional to the quantity of mitochondrial suspension taken for assay (Fig. 4).

Assay procedure applied to various tissues. A variety of plant tissues lacking chlorophyll was assayed according to the procedure described above. The results are summarized in Table 1. The cytochrome oxidase activity of the untreated mitochondria varies over a wide range from 0.6 for pear to 88 for *Ricinus* endosperm. Several factors may be concerned here. In the first place, the figures undoubtedly reflect the proportion of cytoplasm to vacuole in the cells: storage tissues and fruits are highly vacuolated and these tissues have the lower activities. Secondly, it must be pointed out that the mitochondria were isolated in a centrifugal field of only 4000 g, which is probably insufficient to bring down all the mitochondria, so that a greater proportion of the total would be isolated from a tissue in which the mitochondria were large than from a tissue with small mitochondria. There may, again, have been differences in the efficiency with which the cells of the various tissues were disrupted in the initial grinding procedure.

However, the most important aspect of Table 1 is independent of the level of activity in the controls: it is that digitonin increases cytochrome oxidase activity in every case and sometimes by a considerable amount. No attempt was made to see

whether the conditions used for treatment of *Phaseolus* mitochondria with digitonin were indeed appropriate for preparations from other plants, and some of the variation in the potentiating power of digitonin (ranging from under 3 to 52) might be taken to indicate that conditions were far from optimum in certain of the tissues. Yet it seems probable that some of this variation reflects true differences in the enzymic architecture of the mitochondria.

Spectrophotometric and manometric procedures compared. In a study of mitochondria isolated from *Arum* spadix (Simon, 1957) it was found that mitochondria disintegrated by mechanical vibration had a greater cytochrome oxidase activity than controls, when activity was measured spectrophotometrically or manometrically with ascorbic acid as reducing agent. Attempts to repeat this result in manometric experiments with *p*-phenylenediamine as reducing agent were unsuccessful, the disintegrated mitochondria having no more activity than the controls. It was suggested that *p*-phenylenediamine might inhibit the action of the cytochrome oxidase revealed by disintegration.

It seemed particularly desirable to make comparable measurements on the *Phaseolus* mitochondria after treatment with digitonin. However, these mitochondria have an ascorbic acid oxidase (E. W. Simon, unpublished work), so that ascorbic acid is not a suitable reducing substance for use in the manometric determinations; quinol was used instead. The results of three experiments are presented in Table 2.

Considering first the untreated controls, it will be noted that manometric determinations with *p*-phenylenediamine give higher cytochrome oxidase activities than the other procedures, and that increase in the concentration of cytochrome c

Table 1. *Effect of digitonin treatment on mitochondrial cytochrome c oxidase*

Tissue	Cytochrome oxidase (μ l. of O ₂ /hr./g. wet wt.)		Ratio: treated/ control
	Control	Digitonin- treated	
Stem: potato tuber	2.0	51	25
Roots: swede	3.5	38	11
carrot	0.8	9.8	12
beetroot	3.5	24	6.9
leek	23	64	2.8
Fruits: marrow	2.4	80	33
pear	0.6	31	52
Leaves: celery petiole	6.0	110	18
onion bulb	1.2	53	44
cabbage bud	10	120	12
Seedlings: <i>Phaseolus</i> hypocotyl (5 day)	4.7	130	28
<i>Ricinus</i> endosperm (6 day)	88	540	6.1
Fungus: mushroom stipe	6.4	160	25

Table 2. Comparison of manometric and spectrophotometric assays of cytochrome *c* oxidase in *Phaseolus mitochondria*

Cytochrome oxidase was measured in the presence of either 20 μ M- or 60 μ M-cytochrome *c*. In the manometric determinations 0.03M-*p*-phenylenediamine (PPD) or 0.02M-quinol (Q) or both was used to reduce the cytochrome *c*.

		Cytochrome oxidase (μ l. of O ₂ /hr./g. wet wt.)					
		Manometric				Spectrophotometric	
		20 μ M-Cytochrome <i>c</i>		60 μ M-Cytochrome <i>c</i>		20 μ M-Cytochrome <i>c</i>	
Expt. no.	Reducing agent	Control	Digitonin-treated	Control	Digitonin-treated	Control	Digitonin-treated
1	PPD	26	28	32	28	6	110
2	Q	15	74	26	82	5	130
3	PPD	38	37	—	—	6	150
	Q	16	70	—	—	—	—
	PPD + Q	26	60	—	—	—	—

causes a correspondingly smaller increase in activity. These differences can be related to the power of *p*-phenylenediamine to penetrate into the particles and reduce the endogenous cytochrome *c* (Slater, 1949; Fritz & Beevers, 1955). It is clear that cytochrome oxidase activity is increased by treatment with digitonin if it is measured spectrophotometrically or manometrically with quinol as reducing agent, but not when *p*-phenylenediamine is used. Since the two reducing agents together reveal at least some increase in activity after treatment with digitonin (Table 2, Expt. no. 3) it seems that *p*-phenylenediamine may be not so much an inhibitor of the internal cytochrome oxidase as an ineffective reducing substance.

Even the manometric experiments with quinol do not reveal as large an activity in the treated mitochondria as do spectrophotometric measurements. One possible reason is the following. In spectrophotometric experiments samples of mitochondrial suspension were incubated with digitonin for 30 sec., and the action of digitonin was then terminated by the 300-fold dilution on assay; but in manometry 0.5 ml. of the mixture of mitochondria and digitonin was pipetted into a Warburg flask, where it suffered only sixfold dilution, leaving the digitonin concentration high enough to cause a continued decay in cytochrome oxidase activity (cf. Figs. 1, 2). It was found that reduction in the volume of suspension pipetted into the Warburg flask from 0.5 to 0.05 ml. increased the activity/ml. nearly three times. Nevertheless, manometric experiments never yielded so great an increase in activity after treatment with digitonin as did spectrophotometric runs, the highest increase recorded being only eightfold.

Treatment of mitochondria with digitonin compared with other treatments. The treatment of mitochondria with digitonin by the procedure described above revealed a higher cytochrome oxidase

activity than any other treatment investigated (Table 3). Deoxycholate, tauroglycocholate and dodecyl sodium sulphate used under the same conditions as digitonin were only about half as effective; it was noted that prolonged incubation with the bile salts, as with digitonin (Fig. 1), reduced cytochrome oxidase activity. Cetyltrimethylammonium bromide, a cationic detergent, proved to be inhibitory.

Treatment with *Crotalus* venom gave a marked increase in cytochrome oxidase activity: with trypsin there was a slight increase only.

Of physical treatments, the most effective was transfer to hypotonic conditions. Mitochondria washed in water or 0.05M-phosphate instead of the usual sucrose-containing medium had about one-third of the activity of a sample treated with digitonin. Mechanical vibration with Ballotini spheres was yet less effective, while incubation at 25° and two cycles of freezing followed by thawing were quite ineffective.

Effect of medium used in preparing the enzyme system on digitonin treatment. The fact that transfer of mitochondria prepared in a sucrose-containing medium to one of lower molarity was sufficient to increase cytochrome oxidase activity (Table 3) suggested that it would be of interest to compare the action of digitonin on mitochondria prepared in buffered sucrose with its action on particles prepared in a medium lacking sucrose. The results in Table 4 show that cytochrome oxidase activity of controls is higher in the particles prepared in buffer than in those prepared in buffered sucrose, an observation in accordance with the usual practice of homogenizing tissues in plain buffer for cytochrome oxidase assay. However, digitonin treatment quite clearly increases cytochrome oxidase activity in both types of preparation. The highest activities are found in preparations made in buffered sucrose and then treated with digitonin.

Table 3. *Digitonin treatment compared with certain other treatments on Phaseolus mitochondria*

Chemical substances and trypsin were all applied to the mitochondrial suspension at 20 mg./ml. for 30 sec. *Crotalus* venom was made up as a 10% (w/v) solution, 0.1 ml. of this being incubated with 1 ml. of mitochondrial suspension for 30 sec. Mechanical vibration consisted of shaking 3 ml. of mitochondrial suspension with 3 g. of Ballotini spheres at 500 cyc./sec. for 3 min. Pretreatment with phosphate or water was effected by washing the mitochondria in these media instead of the usual sucrose- and phosphate-containing solution.

Chemical	Treatment	Cytochrome oxidase (μ l. of O_2 /hr./g. wet wt.)		Ratio: treated/ control
		Control	Treated	
Digitonin		6.4	120	19
Deoxycholate		9.4	85	9.0
Tauroglycocholate		6.8	59	8.7
Dodecyl sodium sulphate		4.5	27	6.0
Cetyltrimethylammonium bromide		5.5	0	0
Enzymic				
Trypsin		4.5	7.4	1.6
<i>Crotalus</i> venom		5.8	33	5.7
Physical				
Freezing and thawing (twice)		6.4	8.5	1.3
Incubation at 25° for 1 hr.		6.4	4.9	0.8
Mechanical vibration		3.7	8.5	2.3
Pretreatment for 20 min. with 0.05M-phosphate		4.5	28	6.2
Pretreatment for 20 min. with water		7.4	38	5.1

DISCUSSION

It has been shown above that treatment of mitochondria derived from a variety of plant tissues with digitonin results in a pronounced increase in cytochrome oxidase activity, and preliminary studies with particles prepared from rat liver and kidney show that digitonin has the same action on them. Wainio & Aronoff (1955) have shown spectrophotometrically that digitonin (and some other surface-active agents) increase cytochrome oxidase activity in the Keilin and Hartree heart-muscle preparation. Gamble & Lehninger (1956), on the other hand, report a 50% inhibition of the cytochrome oxidase in rat-liver mitochondria by their treatment with digitonin. This result is surprising in view of the studies by Wainio and his colleagues and of the present experiments, but the procedures of Gamble & Lehninger differ from those described above in two respects which might each lower cytochrome oxidase activity: (1) the mitochondria were incubated with digitonin for as long as 20 min., and (2) cytochrome oxidase was measured by the manometric method with ascorbic acid as reducing agent.

Mechanism of action of digitonin

In an earlier paper (Simon, 1957) the increase in cytochrome oxidase activity brought about in *Arum* mitochondria by mechanical vibration and by (prolonged) treatment with digitonin was cited as evidence for a dual location of cytochrome oxidase, distinction being drawn between a superficial location accessible to exogenous cytochrome c

and a second position which is internal and inaccessible. It was suggested that these two locations might be respectively the outer envelope of the mitochondria and the cristae or villi revealed by electron-microscope studies. So long as the mitochondria were intact exogenous reduced cytochrome c would assay only the oxidase located at the surface; to measure the activity of the internal oxidase it would be necessary first to puncture the outer envelope. This simple picture now seems incorrect, since (1) if it were true one would expect that mechanical vibration and treatment with digitonin would be about equally effective, whereas in fact vibration is a relatively inefficient treatment (Table 3), and (2) the particles prepared in plain buffer lack many of the integrated activities of the intact mitochondria and consist of mitochondrial fragments (Gamble & Lehninger, 1956), yet treatment with digitonin increases cytochrome oxidase activity in these already fragmented mitochondria (Table 4).

The fact that several other surface-active agents besides digitonin are effective in increasing cytochrome oxidase activity (Table 3 and Wainio & Aronoff, 1955) leads to the suggestion that they may act by disorganizing lipoprotein structures, and specifically by freeing cytochrome oxidase molecules from their normal close association with other large molecules. Ball & Cooper (1949) first suggested that lipids were important as structural elements in mitochondria, and Green (1956) has pictured adjacent cytochrome oxidase chains as being held together in a close-knit framework by lipid in such a way that dispersal of the lipid would

Table 4. *Effect of medium used in preparing mitochondria on cytochrome c oxidase*

Mitochondria were isolated by centrifuging at 10 000 *g*. Each value is the mean of two or three determinations.

Material	Cytochrome oxidase (μ l. of O ₂ /hr./g. wet wt.)			
	Mitochondria prepared in 0.05M-phosphate		Mitochondria prepared in 0.05M-phosphate, 0.2M-sucrose	
	Control	Digitonin- treated	Control	Digitonin- treated
Potato tuber	33	100	20	260
Mushroom stipe	34	120	24	280
<i>Phaseolus</i> hypocotyl	170	390	74	530

free the individual cytochrome chains and render cytochrome oxidase accessible to exogenously supplied substrate. Mechanical vibration would be a poor means of effecting so subtle a disintegration of mitochondrial structure.

The enhancement of cytochrome oxidase activity after transfer of the mitochondria to hypotonic conditions or resulting from preparation in a medium of low tonicity is presumably due to swelling of the lipoprotein framework. It will be seen from Table 4 that although digitonin stimulates the cytochrome oxidase of preparations made in plain buffer the degree of stimulation is relatively small, presumably because the particles prepared in this way are already disintegrated to some degree. If, as seems likely, the normal procedure for preparing mitochondria in buffered sucrose yields some fragmented mitochondria as well as intact ones, then centrifuging at 10 000 *g* would isolate a preparation having more fragments than a preparation made at 4000 *g*, and it would show the smaller increase on treatment with digitonin as was indeed found (cf. Tables 1 and 4).

It was observed in the present experiments that mitochondria lost the power to oxidize succinate when treated with digitonin although cytochrome *c* was oxidized at a faster rate. Comparable inhibitions of succinoxidase by bile salts and other surface-active agents and by venom have been reported earlier (see review by Wainio & Cooperstein, 1956); and it is known that whereas exposure to hypotonic conditions reduces mitochondrial succinoxidase it has little effect on succinic dehydrogenase and may increase cytochrome oxidase (Hogeboom, Claude & Hotchkiss, 1946; Potter, 1951). It seems that under the appropriate conditions the multi-enzyme succinoxidase system is disintegrated in such a way as to leave the cytochrome *c* oxidase still functional. The results presented in Fig. 1 suggest that cytochrome oxidase itself is attacked if the particles are treated with digitonin for long periods of time; it would indeed

be surprising if this did not happen in view of the fact that cytochrome oxidase has a large lipid content (Marinetti, Scaramuzzino & Stotz, 1957).

It is pertinent to recall here that under the appropriate conditions digitonin can set free from mitochondria a system which retains the capacity for oxidative phosphorylation (Cooper & Lehninger, 1956) and from chloroplasts, a system with cytochrome *c* photo-oxidase activity (Nieman & Vennesland, 1957). Further, digitonin, like bile salts, has the power to clarify mitochondrial suspensions (Wainio & Aronoff, 1955; Witter & Cottone, 1956).

The ability of trypsin to increase cytochrome oxidase activity is presumably a result of digestive attack on mitochondrial proteins. The activity of *Crotalus* venom is of interest since venoms contain a variety of toxins and enzymes, including a phospholipase A (Slotta, 1955). Nygaard (1953) has examined the effect on rat-liver mitochondria of a crystalline phospholipase A prepared from venom of *C. terrificus terrificus* and finds that succinic dehydrogenase is inhibited less than succinoxidase, whereas cytochrome oxidase activity may be increased (Nygaard & Sumner, 1953). The phospholipase A of venom is very stable to boiling; in the present experiments it was found that venom boiled for 15 min. increased cytochrome oxidase only about one-third as much as the unboiled venom. In contrast with these results, Braganca & Quastel (1953) observed 50 % or more inhibition of the cytochrome oxidase activity of a rat-brain homogenate after treatment with heated cobra venom, and somewhat similar results are reported by Edwards & Ball (1954). But examination of the methods used shows that in both investigations the venom was allowed to act on the enzyme preparation for 30 min. or more, and that cytochrome oxidase activity was measured manometrically with *p*-phenylenediamine as reducing agent; both of these conditions would tend to obscure any stimulation of cytochrome oxidase.

Assay of cytochrome c oxidase

The assay of cytochrome oxidase presents two special problems. The first, already discussed by Slater (1949) and by Smith & Conrad (1956), concerns the effect of cytochrome *c* concentration on the activity of the enzyme. The second, which is considered here, is the question of the ease of access of the substrate to its enzyme. It is clear from Table 4 that low cytochrome oxidase values are given by particles prepared in sucrose-containing media and that preparation in hypotonic media or treatment with digitonin give successively higher values. The interpretation put upon these results is that digitonin treatment releases cytochrome oxidase molecules from close association with neighbouring macromolecules. At the same time, as discussed above, it seems that digitonin actually destroys some cytochrome oxidase activity, so that the treatment underestimates the enzyme activity of the preparation—and that by an unknown amount. However, the value of the treatment with digitonin is that it gives a better approximation to the true activity than does assay of untreated particles.

Finally it should be said that although the experiments described above deal only with treatment of mitochondrial preparations with digitonin, the treatment should have a wider application than this, for instance to tissue homogenates or to other particles suspected of bearing cytochrome oxidase.

SUMMARY

1. The cytochrome *c* oxidase activity of suspensions of plant mitochondria is increased by incubating them with 20 mg. of digitonin/ml. for 30 sec. Increases ranging from 2.8 times to 52 times were recorded with mitochondria isolated from various plant tissues.

2. Cytochrome oxidase was measured spectrophotometrically. In manometric experiments, rather smaller increases in activity were found when quinol was used as reducing agent, but with *p*-phenylenediamine there was no increase at all.

3. Treatment with digitonin was more effective than were various other chemical, physical and enzymic treatments.

4. It is suggested that digitonin acts by dispersing lipid links between protein molecules in the

mitochondria in such a way as to render cytochrome oxidase molecules more accessible to their exogenous substrate, reduced cytochrome *c*.

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