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Preparation and Properties of Sweet Potato Mitochondria [1, 2]

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The object of this paper is to describe in detail the preparation, oxidative capacities, and response to various inhibitors of plant mitochondria that can be described biochemically as reasonably intact. The isolation of plant mitochondria which fulfill the suggested requirements for respiratory control (12) has been reported previously only from this laboratory (5) but the procedure was not given in detail.

The sweet potato, *Ipomea batatas*, is an object obtainable from local markets for some months during the year and one from which mitochondria are easily prepared. In spite of the recent and thorough studies of Hackett et al. (22) and of Baker and Lieberman (3), it was thought that a study of the preparation and oxidative capacities of tightly coupled sweet potato mitochondria was necessary. This paper describes the preparation and properties of such mitochondria. In addition, some observations on white potato, *Solanum tuberosum*, and on skunk cabbage, *Symplocarpus foetidus*, are included.

Methods

Preparation of mitochondria. Sweet potatoes from local markets were peeled and refrigerated before use; all operations were carried out between 0° and 4°. Sweet potato tissue (300 g) was grated into 600 ml of chilled medium, containing 0.25 M sucrose, 0.37 M mannitol, 4 mM cysteine, and 5 mM EDTA[4]. The mixture was blended at low speed (60 volts) for 20 seconds and then at full speed for 2 to 3 seconds in a Waring blendor. During the blending the pH of the suspension was monitored (narrow range indicator paper) and maintained between 7.2 and 7.5 by dropwise additions of 5.5 M KOH. The homogenate was squeezed through muslin and the filtrate was centrifuged at 1,500 × *g* for 15 minutes and the precipitate discarded. The supernatant suspension from this centrifugation was centrifuged at 10,000 × *g* for 15 minutes and the pellet was washed with a mixture of 0.25 M sucrose and 0.37 M mannitol

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[4] Abbreviations: BSA, bovine serum albumin; DNP, dinitrophenol; EDTA, ethylenediaminetetraacetate; HOQNO, 2-n-heptyl-4-hydroxyquinoline-N-oxide; P.A., mixture of 0.8 mM N,N.N′,N′-tetramethyl-p-phenylene diamine and 4 mM sodium ascorbate.

and resedimented by centrifuging at 10,000 × *g* for 15 minutes. The resulting pellet was carefully suspended in 0.5 M mannitol so that a minimal amount of starch was resuspended and then recentrifuged. The final mitochondrial pellet was suspended in 0.5 M mannitol.

Mitochondrial fractions from potato tubers, *Solanum tuberosum,* were prepared as described above for sweet potato, except that it was not necessary to blend at a high speed and 0.1 % BSA was added up to and including the final sedimentation.

Oxygen utilization from the mitochondrial suspensions was measured polarographically in an air-saturated medium with either a vibrating platinum micro-electrode or a Clark electrode. The standard reaction medium in which mitochondria were tested contained 0.3 M mannitol, 0.01 M tris buffer, pH 7.2, 0.1 mM EDTA, 0.01 M potassium phosphate, and 5 mM magnesium chloride. Measurements were made at room temperature. All of the data obtained were calculated to the nitrogen content of each mitochondrial preparation. The mitochondrial nitrogen was assayed by nesslerization following acid digestion.

Results and Interpretation

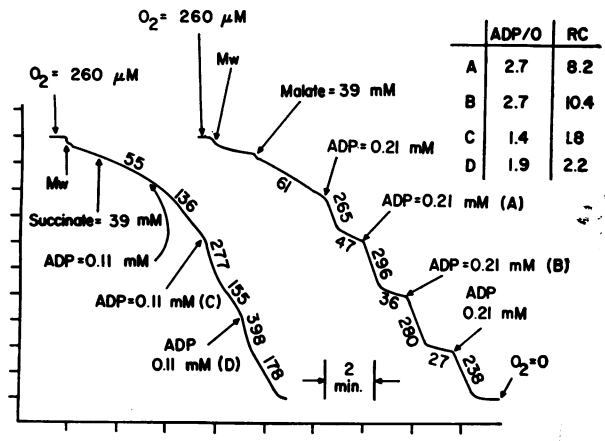
I. *Preparation.* The technique of grating chilled tissue and blending at low speed with a very short blending period disrupted the tissue and allowed the release of mitochondria without any apparent damage to their biochemical integrity. A wide survey of isolation media was made. The mixture finally employed was chosen because it allowed maintenance of a suitable osmotic concentration and density. Mannitol, the last wash medium, allowed an easier separation of the mitochondria from the starch in the final precipitate than did sucrose (5). However it is possible to prepare mitochondria, as described here, in a sucrose medium if great care is taken in resuspending the precipitates. This latter procedure usually resulted in lower yields.

The use of strong buffers has been avoided and maintenance of the pH at 7.2 to 7.5 by dropwise additions of KOH proved sufficient. It was found that pH was an extremely critical factor when isolating mitochondria from sweet potato tissue (cf. 5). When isolated at a pH of 8 or higher the particles showed very little oxidative activity and did not respond to ADP. Slightly acid conditions (pH 6.5) of isolation had similar deleterious effects. However, mitochondria isolated from sweet potato tissue at the pH resulting from disruption of the tissue behaved as DPNH oxidase particles showing respiratory control; other substrates were not oxidized by this type of preparation.

Beneficial effects of adding cysteine to the isolation medium have been reported (20), however excessive use of this compound is not recommended (37). Here it was found that concentrations of cysteine greater than 10 mM resulted in preparations with low activity, but it is true that some tissues require more cysteine than do others. An inert protein, e.g. BSA, has been used to stabilize plant preparations (15, 31, 35). Active preparations from white potato tissue were obtained more consistently when BSA was included in the isolating medium.

In contrast to most mitochondrial preparations isolated from plant tissues, the mitochondria described here showed no response, in their oxidative capacities, to added cytochrome c or added DPN.

II. *Oxidation and Coupling.* The oxidative and phosphorylative activities of the sweet potato and white potato mitochondria are shown by the polaro-



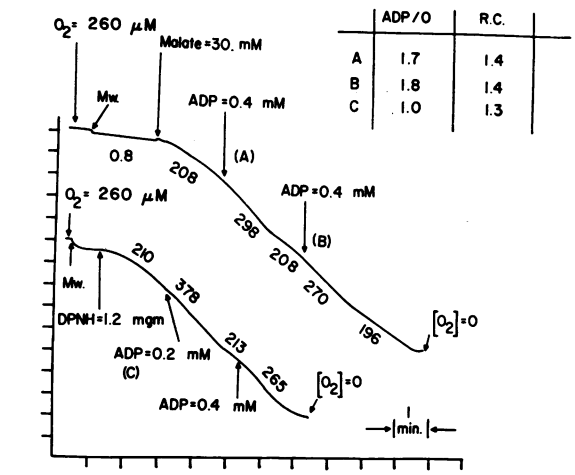


FIG. 1. Polarograph traces showing the respiratory and phosphorylating activities of sweet potato mitochondria (M_W). Assayed in 2.4 ml standard reaction medium containing 298 μg mitochondrial nitrogen. Additions are shown as final concentrations. Rates of O_2 utilization are expressed as mμ atoms per minute. The ADP/O and respiratory control (R.C.) ratios are also given. R.C. = state 3 rate/state 4 rate.

FIG. 2. The oxidative and phosphorylative activities of mitochondria (M_W) isolated from fresh white potatoes. Assayed in 3.0 ml standard reaction medium containing 356 μg mitochondrial nitrogen. Additions are shown as final concentrations. Rates of O_2 utilization are expressed as mμ atoms per minute. ADP/O and respiratory control (R.C.) ratios are also given.

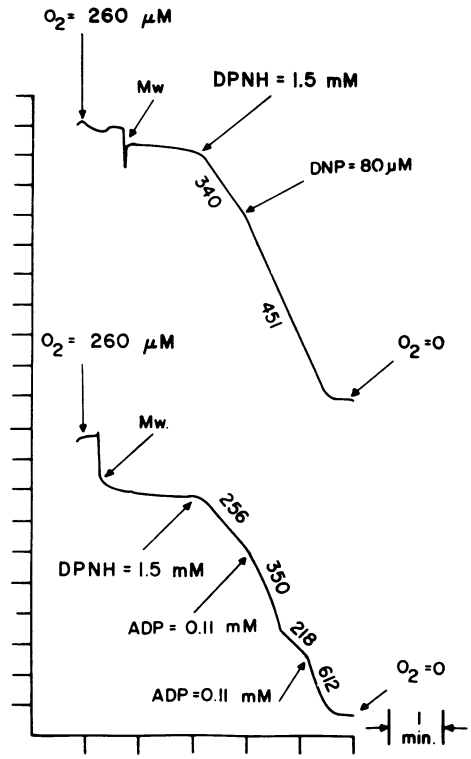
graphic records in figures 1 and 2. Figure 1 shows that the oxidation of both succinate and malate was stimulated by the addition of ADP. Furthermore, it can be seen that the rate of substrate oxidation decreased when all of the ADP had been phosphorylated, and further additions of ADP stimulated oxidation of substrate. Following the nomenclature of Chance and Williams (10) the ADP-stimulated rate is referred to as state 3 and the ADP-limited rate as state 4. It can also be seen from figures 1 and 2 that the O_2 utilization by endogenous substrates is very small. The ADP/O ratios, calculated according to Chance and Williams (10) are very close to the accepted theoretical P/O maxima (fig 1).

The data obtained with white potato mitochondria (fig 2) show similar results although the degree of respiratory control was small. However, extraction of active mitochondria from white potato tissue has been difficult (21) and the low degree of control may even be due to factors precipitated with the mitochondria (5).

The sweet potato and white potato mitochondria also show respiratory control with DPNH as substrate (fig 2). That this is due to a true phosphorylation is shown in figure 3 where DNP (8×10^{-5} M) replaced ADP in stimulating oxidation but without any evidence of control. This latter evidence shows that the mitochondria are coupled to phosphorylation. The ADP/O ratios with DPNH as substrate (fig 2) deviated from the theoretical maximum. However, under the conditions used, large amounts of DPNH were added and consequently there was uncoupling (27).

In addition to ADP/O ratios, indicating the degree of uncoupling, it is also possible to determine a respiratory control ratio (R.C. = state 3 rate/state 4 rate). The respiratory control ratio for sweet potato mitochondria (fig 1) was severalfold greater with malate than with succinate as substrate. This difference is also reflected in the sharp cut-off obtained with malate on the exhaustion of ADP (fig 1). Similar differences between succinate and DPN-linked substrates have been obtained with mitochondria isolated from animal tissues (8).

The respiratory control ratio, as a measure of the degree of coupling, indicates the leakage of electron transfer without concomitant phosphorylation. However, should some adenosine triphosphotase be present allowing ADP to recycle slowly, the state 4 rate may not be a true measure of the nonphosphorylating oxidation rate. This possibility can be tested by using oligomycin which inhibits phosphorylation of animal systems without uncoupling (14, 26). Figure 4 shows that oligomycin inhibited the oxidation of succinate and that the ratio of this inhibited rate to that of state 3 is the same as the respiratory control ratio. However, this was not a consistent observation (see table I). Figure 4 also shows that oligomycin inhibited oxidation by inhibiting phosphorylation, an inhibition that was released on addition of DNP, in other words, the system was coupled until the addition of DNP.



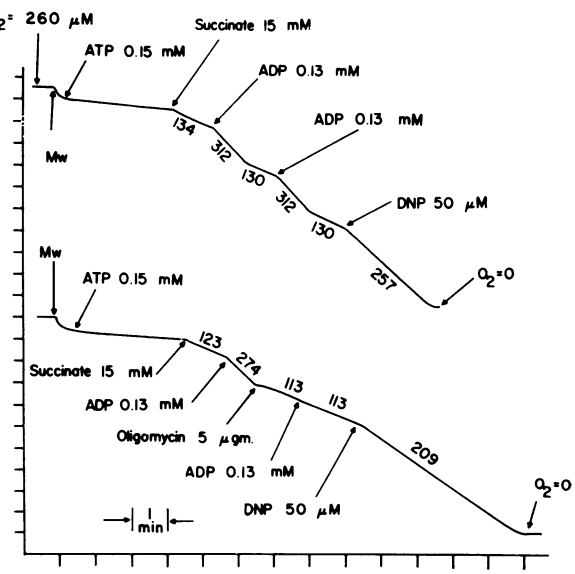


FIG. 3. The effects of ADP and DNP on DPNH oxidation by sweet potato mitochondria (M_W). Assayed in 2.0 ml standard reaction medium containing 295 μg mitochondrial nitrogen. Additions are shown as final concentrations. Rates of O_2 utilization are expressed as mμ atoms per minute.

FIG. 4. The effect of oligomycin on succinate oxidation of potato mitochondria (M_W). Assayed in 3.3 ml standard reaction medium containing 179 μg mitochondrial nitrogen. Additions are shown as final concentrations. Rates of O_2 utilization are expressed as mμ atoms per minute.

Table I

Effect of Various Treatments on the Rate of Succinate Oxidation

Rates are expressed as mμ atoms O_2/minute. Initial rates are derived from the first minute of reaction. Assayed in 3.3 ml standard reaction medium, 278 μg mitochondrial nitrogen. Additions are shown as final concentrations.

	Succinate 15 mM	ADP 0.15 mM	ATP 0.17 mM	DNP 50 μM	Oligomycin 5 μg	Rates		T max*** Minutes	Rates	
						Initial	Maximum		State 3 ADP 0.15mM	State 4
	+	−	−	−	−	57	180**	8.0		
A*	+	+	−	−	−	66	255	3.0	416	
	+	−	+	−	−	72	255	2.0	410	
		+	−	−	−	88	261	2.5	403	249
		−	+	−	−	192	252	0.5	422	249
		−	−	+	−	38	151**	13.0		
B*		+	−	+	−	69	340	6.0	340	
		−	+	+	−	221	346	3.0		
		+	−	−	+	76	192**	6.5		
		−	+	−	+	192	192	0		

* A, Compounds added with sodium succinate at zero time. B, Compounds added 3 minutes before succinate.
** — Maximum rate attained before O_2 = O.
*** — Time taken to attain maximum rate after addition of sodium succinate.

III. *Uncoupling and Inhibition by DNP.* It is also clear from figure 4 that, in the presence of oligomycin, DNP (5×10^{-5} M) did not restore the oxidation rate to the level of the state 3 rate. A series of experiments with different DNP concentrations is shown in figure 5. The potato mitochondria were incubated with ATP and amytal for 3 minutes, then allowed to oxidize succinate for 2 minutes before the addition of ADP. After the subsequent state 4 rate had been determined, the particular concentration of DNP was added. Amytal was included to inhibit the oxidation of substrates with DPN-linked dehydrogenases. The results of 2 separate series of experiments are presented in figure 5. In both cases the optimal DNP concentration for maximal stimulation was found to be between 40 and 50 μM.

At low concentrations of DNP further additions of ADP had an enhancing effect on oxidation, whereas at higher DNP concentrations further ADP additions had no effect. Thus at low concentrations of DNP the stimulation of oxidation was small due to incomplete uncoupling, but at high concentrations the inhibitory effect of DNP became evident.

IV. *Oxalacetate Inhibition of Succinate Oxidation.* In figure 1 the initial rate of succinate oxidation by sweet potato mitochondria was significantly lower than the state 4 rate. Moreover, the first ADP addition did not produce a rapid stimulation of oxidation (the state 4 rate of oxidation was not attained). However, on the second addition of ADP a rapid stimulation of oxidation occurred and this rate eventually decreased to the ADP-limited rate. Such an induction period for the ADP stimulation of succinate oxidation has already been observed with cauliflower mitochondria (5). This induction period which was not evident with malate nor with subsequent additions of ADP (fig 1) could not be a reflection of a slow penetration of ADP into the mitochondria. Furthermore, the addition of DNP instead of ADP did not stimulate succinate oxidation, whereas adding DNP after ADP treatment did stimulate oxidation (fig 4). Thus it appears that during the initial phase of succinate oxidation ADP is not the factor limiting oxidation.

The white potato mitochondria behaved similarly as shown in figure 6 where the first addition of ADP produced an oxidation rate somewhat in excess of the state 4 rate. The maximum rate of succinate oxida-

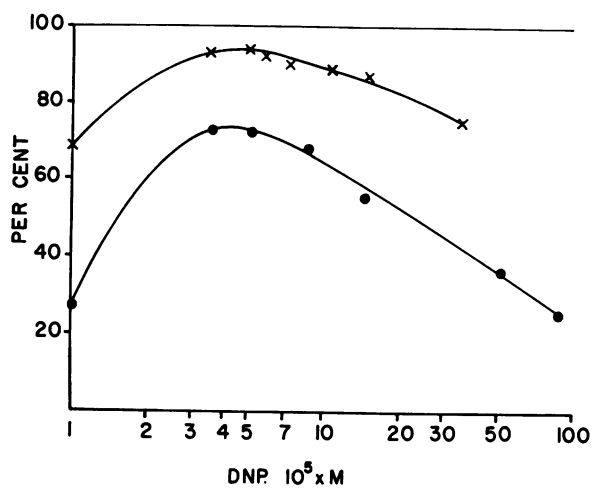


FIG. 5. A semilogarithmic plot showing the effect of DNP on the rate of succinate oxidation of potato mitochondria (M_W). × —— × DNP rate expressed as a percentage of the state 3 rate (100%), 278 μg mitochondrial nitrogen. ○ —— ○ DNP rate expressed as a percent stimulation of the state 4 rate $\frac{\text{DNP rate} - \text{state 4 rate} \times 100}{\text{state 4 rate}}$, 313 μg mitochondrial nitrogen. Assay conditions: 3-3 ml standard reaction medium, 0.17 mM ATP, 15 mM sodium succinate, 0.15 mM ADP and 2 mM amytal.

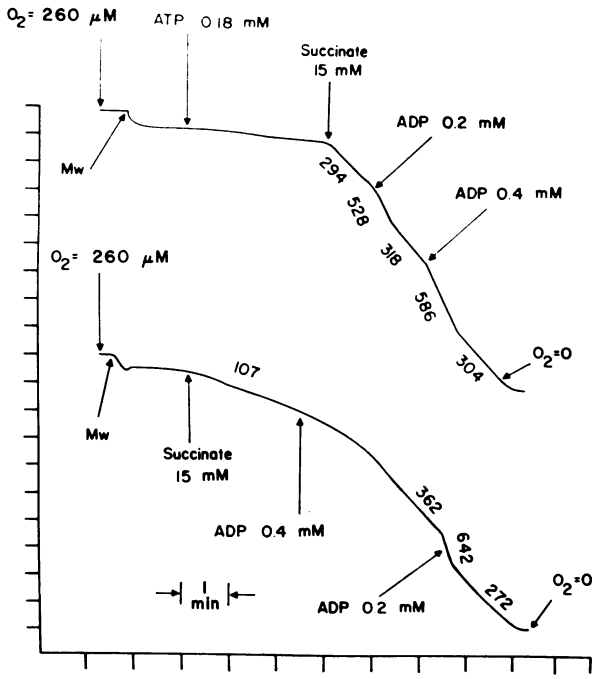


FIG. 6. The effect of preincubation with ATP on succinate oxidation by potato mitochondria (M_W). Assayed in 3.0 ml standard reaction medium containing 360 μg mitochondrial nitrogen. Additions are shown as final concentrations. Rates of O_2 utilization are expressed as mμ atoms per minute.

tion attained after the first ADP addition depended on the initial concentration of the ADP.

Penetration of succinate into the mitochondria could be the cause of the induction period. However, after incubation with ATP, the mitochondria responded rapidly to succinate (fig 6). Apparently ATP is necessary to elicit maximal rates of succinate oxidation. Hence, succinate treated mitochondria (fig 1, 6) apparently require ATP for the initial stimulation of oxidation. Since the incubation period with ATP was arbitrarily standardized to 3 minutes, the initial high rate of succinate oxidation was detected only in those preparations for which this incubation period would elicit the maximal rate of succinate oxidation. Many preparations did not show this initial effect and in figure 6 the ATP preincubation did not elicit an immediate succinate rate equal to that of state 4.

Table I shows that the stimulation of succinate oxidation was due to the presence of ATP. In the presence of succinate this stimulation developed maximal oxidation rate slightly faster than that produced by ADP. In neither instance was the initial rate much faster than the control (table IA). However, the highest rate developed before anaerobiosis was significantly higher than the control and the state 3 rate could be attained with further additions of ADP. The progressive increase of O_2 utilization with succinate alone may be due to the formation of endogenous high energy intermediates resulting in ATP formation. In fact DNP had an inhibitory effect (table IB) on the initial and maximal rate of succinate oxidation. This inhibition is not to be confused with the inhibitions observed in figure 5 where DNP inhibited the state 3 rate.

Preincubation with ADP produced the same maximal rate (state 4) as did ATP (table IB), but stimulated only slightly the initial rate of oxidation. This slight stimulation of the initial rate may have been due to some phosphorylation of ADP during oxidation of endogenous substrate, as both oligomycin and DNP had inhibitory effects. However, ATP could also be formed from ADP by an adenylate kinase reaction. Also, the time taken to develop the maximum rate after incubation with ADP was not very different from that when the mitochondria were not incubated with ADP (table IA).

Dinitrophenol added with ATP had no effect on the initial rate of oxidation, but elicited a maximum rate faster than that with ATP alone. This maximum rate would be expected from consideration of uncoupling and of the state 3 rate (fig 5). The same maximum rate was obtained when the mitochondria were incubated with ADP and DNP. This may seem inconsistent with the hypothesis of ATP being essential and appears inconsistent with the results obtained with succinate and DNP alone. However, the concentration of DNP used (50 μM) gave maximum rates of oxidation but not necessarily the maximum degree of uncoupling.

Hackett et al. (21) found that 1×10^{-4} M DNP decreased the P/O ratio of white potato mitochondria oxidizing succinate but as seen here, this concentration of DNP markedly inhibited this oxidation. In the presence of oligomycin and ADP or ATP the maximum rate obtained was even lower than the state 4 rate, an observation suggesting that some hydrolysis of ATP was occurring under state 4 conditions, and that oligomycin was more effective in preventing phosphorylation of ADP than was DNP. Thus oligomycin did not affect the initial rate of oxidation after preincubation with ATP, but did not allow the expected maximum rate to develop. The development of the maximum rate with both oligomycin and ADP suggests that either ATP was formed by an adenylate kinase reaction or that some factor other than ATP is effective in attaining the maximal rate. However, consideration of the periods taken to obtain the maximum rate indicates that even if ATP is not the sole factor involved, it is certainly the most effective factor.

The results of table I, showing an ATP requirement for succinate oxidation, are similar to those reported for rat liver mitochondria pretreated with arsenate and DNP (2), a result interpreted as an oxaloacetate inhibition of succinate dehydrogenase (13). It is known that oxaloacetate can inhibit the succinate oxidation of plant mitochondria (1), here confirmed, and of animal particulates (30).

The succinate dehydrogenase of the mitochondria may have been severely inhibited by oxaloacetate. In fact, the formation of oxaloacetate within the mito-

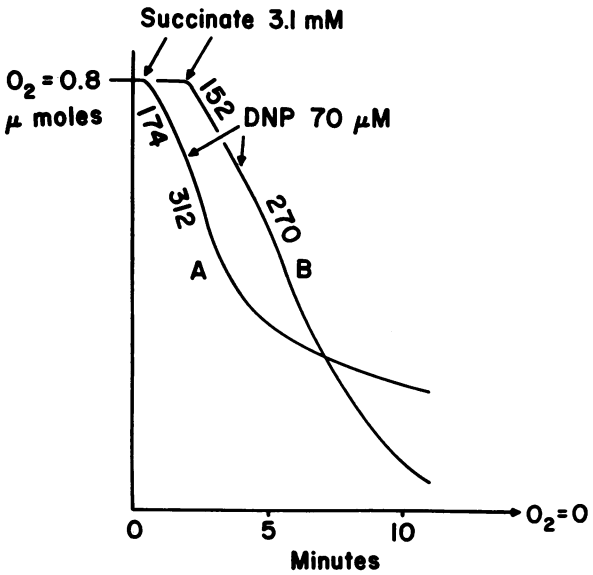


FIG. 7. The effect of amytal on succinate oxidation of sweet potato mitochondrial (M_W). (A) Assayed in 3.0 ml standard reaction medium containing 0.16 mM ATP. Additions are shown as final concentrations. (B) As A and containing 2.0 mM amytal. Rates of O_2 utilization are expressed as mμ atoms per minute.

chondria can inhibit succinate dehydrogenase. Sweet potato mitochondria preincubated with ATP oxidized dilute concentrations of succinate (fig 7A); the addition of DNP stimulated this rate but a marked inhibition developed with time. However, if the experiment was repeated with amytal in the incubation medium (fig 7 trace B) the inhibition developed more slowly. Amytal would prevent oxaloacetate formation by inhibiting malate oxidation. The lower rates of succinate oxidation in the presence of amytal are also a reflection of the inhibition of malate oxidations. The decrease in rate of succinate oxidation in figure 7 (trace B) may even be due to succinate becoming rate limiting. Similar effects were obtained by treating the mitochondria with small amounts of malate before adding succinate.

In rat liver mitochondria, inosine triphosphate was as effective as ATP in overcoming the oxaloacetate inhibition (13). However, with the plant mitochondria only the adenine nucleotides were found to be effective.

Oxaloacetate is a competitive inhibitor of succinate dehydrogenase (30). The nature of the inhibition was studied here with white potato mitochondria and the double reciprocal plot is shown in figure 8. Both series were incubated with amytal and DNP but series A also had ATP present. The nature of the inhibition appears to be noncompetitive. However, the rates in figure 8 represent initial rates and if the oxaloacetate-enzyme complex dissociates slowly (13), it may not be possible to demonstrate competitive inhibition from the initial rates.

In figure 1 it is not noticeable that the rate of malate oxidation decreased with time, in spite of the high concentration of malate. The progressive inhibition of malate oxidation, probably due to oxaloacetate accumulation, was more noticeable in the presence of DNP. Apparently ATP is quite effective in removing oxaloacetate although it may not be able to cope with fast rates of oxaloacetate formation.

A study on the effect of the components of the reaction medium revealed that EDTA, or P_i, could be omitted without preventing the ATP stimulation of succinate oxidation. When Mg^{++} was omitted from the medium the development of maximum oxidation (in the absence of added ADP) took longer thus implicating Mg^{++} as a cofactor of the system (29). Since EDTA was present in the medium, it is quite possible that this compound removed any trace of Mg^{++} (36).

V. *Studies with Amytal.* The effects of amytal, which completely inhibits oxidation via pyridine-nucleotide linked dehydrogenases in phosphorylating animal mitochondria (11), have not been clearly defined for plant mitochondria. Whereas amytal appears to be an ineffective inhibitor of skunk cabbage (9) and apple (28) particles, it is a potent but not complete inhibitor of beet root particles (38). Jackson et al. (24) have reported amytal inhibition of phosphorylation and respiration in barley root particles, an inhibition observed with succinate as substrate. Contrary to the results of Hackett et al. (22) amytal was found to be an effective inhibitor of sweet potato mitochondria.

Table II shows the inhibition of oxidations of sweet potato mitochondria by amytal. The effects of subsequent additions of ADP and succinate are also shown. We see in column 1 that amytal has little effect on the rate of malate oxidation in the

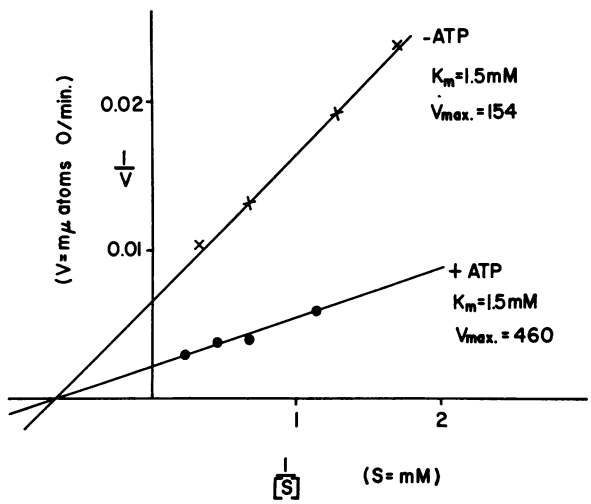


FIG. 8. A double reciprocal plot of the initial rates of succinate oxidation by potato mitochondria, with and without preincubation with ATP. Assayed in 3.2 ml standard reaction medium containing 468 μg mitochondrial nitrogen, 60 μM DNP, 2.0 mM amytal and 0.5 mM ATP where indicated.

Table II

Effects of Amytal on Sweet Potato Mitochondria

Assay conditions: standard reaction medium, 2.0 ml; mitochondrial nitrogen 447 μg per reaction vessel. Rates are expressed as mμ atoms O_2/minute. Figures in parentheses are per cent inhibitions under the conditions specified.

Substrate	Rate					
	Control Rate	Amytal (mM)			ADP (0.26 mM)	Succinate (5 mM)
		1.8	3.6	5.4		
Malate (0.05 M)	112	112 (0)	92 (9)	...	92	92
Malate (0.05 M) + ADP (1.3 mM)	396	200 (49)	76 (83)	40 (90)	...	76
Succinate (5 mM)	80	80 (0)	...	...	292	
Succinate (5 mM) + ADP (1.3 mM)	314	314 (0)	260 (17)	...	...	...

absence of ADP. However neither ADP nor succinate stimulated this amytal rate. However, if ADP was added before amytal to elicit the state 3 rate, similar concentrations of amytal had a marked effect. Again succinate had little stimulative effect on this rate.

Thus an inhibition by amytal could not be demonstrated until the maximum rate of malate oxidation under phosphorylating conditions was attained. Table II also shows that amytal does not inhibit succinate oxidation nor does it prevent the ADP stimulation of succinate oxidation. The small inhibition of succinate oxidation may be indicative of concomitant malate oxidation under these conditions. The inability of succinate to realize any O_2 uptake after malate oxidation was probably due to the accumulation of oxaloacetate.

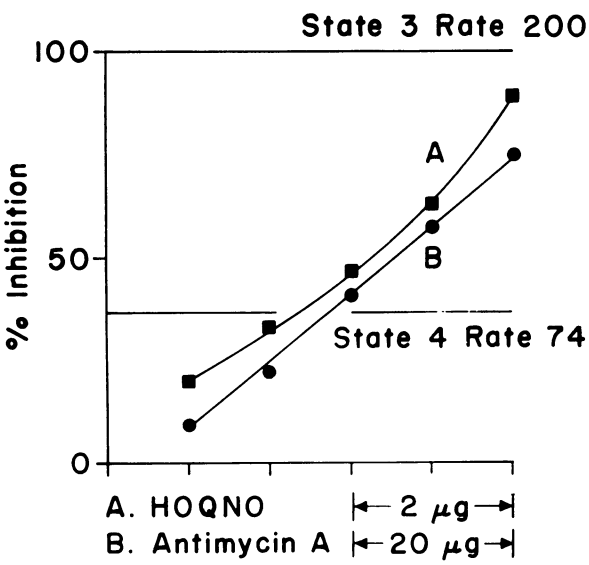


FIG. 9. Effects of Antimycin A and HOQNO on malate oxidation by sweet potato mitochondria. Assayed in 2.0 ml, standard reaction medium, containing 376 μg mitochondrial nitrogen. State 3 and 4 rates are expressed as mμ atoms O_2 per minute.

The white potato mitochondria gave similar results with amytal when malate was used as substrate. However, with DPNH as substrate it was found that amytal was not a potent inhibitor, a result that may have been due to the presence of nonmitochondrial DPNH oxidase (18) or to an extended DPNH oxidation catalyzed by the mitochondria (27). This external system is insensitive to many respiratory chain inhibitors and is stimulated by added DPNH or cytochrome c (38).

VI. *Studies with Antimycin A and HOQNO.* The inhibitory effects of antimycin A and HOQNO are shown in figure 9. The inhibitions parallel one another but concentrations higher than those recorded for plant mitochondria (22, 28) were required. The inhibitor/nitrogen ratios calculated from figure 9 are 8×10^{-3} and 92×10^{-3} for HOQNO and antimycin A, respectively. However, the sensitivity to antimycin varied markedly during the course of this work, an observation that probably reflects variation in the integrity of the mitochondrial membrane among preparations. It is generally agreed that antimycin A penetrates the mitochondrial membrane more slowly than does HOQNO. The mechanism of inhibition remains unknown (17). The loss of antimycin A inhibition caused by degradation of the inhibitor has been reported (28). However, this degradation occurred over a period of several hours, and would not be applicable to the experiments described here.

VII. *Studies with Malonate.* Malonate was found to inhibit succinate oxidation and Honda and Muenster (23) have indicated some of the complications arising from its use. Using the apparent Michaelis constant for succinate of 1.5 mM, the mean affinity constant for malonate inhibition was calculated (16) to be 2×10^{-4} M, assuming a purely competitive inhibition. However, the affinity constants for malonate calculated here and by Honda and Muenster (23) suffer because only O_2 uptake was measured. Neither oxidation nor effects of the subsequent intermediates (e.g. oxaloacetate) were controlled. The calculated values of the constants

Table III

The effect of Cyanide on Succinate and Malate Oxidations of Sweet Potato and Skunk Cabbage Mitochondria

Assayed in 3 ml standard reaction medium. (A) sweet potato mitochondria. (B) skunk cabbage mitochondria. The mitochondria were allowed to oxidize substrate in the presence of excess ADP until the state 3 rate was attained before the inhibitor was added. Values in parentheses are per cent inhibition.

	Substrate	Rates (mμ atoms O_2/min)			
		Control (State 3)	KCN (10^{-5} M)	KCN (10^{-4} M)	KCN (10^{-3} M)
A	Malate (30 mM)	468	...	139 (70)	139 (70)
	Succinate (15 mM)	334	268 (20)	162 (52)	111 (67)
B	Malate (30 mM)	314	242 (23)	227 (28)	227 (28)
	Succinate (15 mM)	468	353 (25)	298 (36)	288 (38)

reported here fall within the range of those reported (23) for lupine mitochondria.

VIII. *Studies with Cyanide.* The cytochrome-oxidase inhibitors, azide and cyanide, even at high concentrations, did not completely inhibit oxidations of malate and succinate by sweet-potato and skunk-cabbage mitochondria. Since there are reports that azide can inhibit substrate dehydrogenases (6) only the effects of cyanide were studied. Table III shows that the mitochondria isolated from 2 cyanide-insensitive tissues show a strong resistance (rather than insensitivity) to cyanide inhibition. Similar results have been reported for other plant mitochondria (19). This effect was further studied with the sweet potato mitochondria.

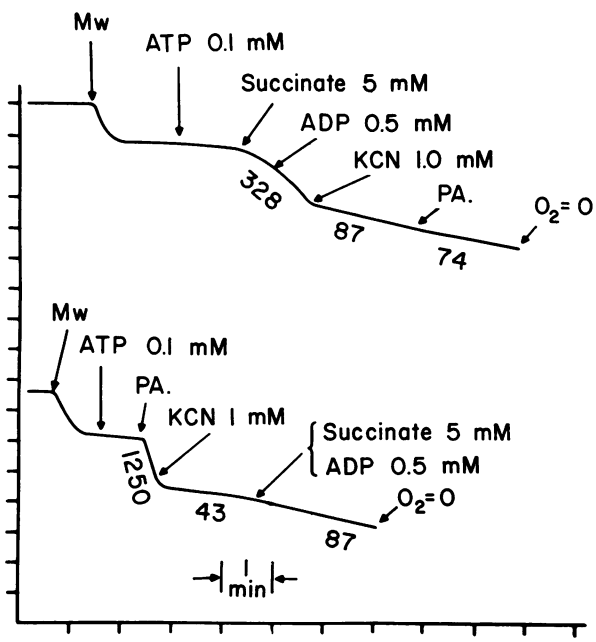


FIG. 10. The effect of substrate on cyanide inhibition of sweet potato mitochondria (M_W). Assayed in 3.0 ml standard reaction medium containing 780 μg mitochondrial nitrogen. P.A.: N,N,N¹,N¹-tetramethyl-*p*-phenylene diamine (0.8 mM) and sodium ascorbate (4 mM). All concentrations are final concentrations. Rates are expressed as mμ atoms O_2 per minute.

The inability of mM cyanide to inhibit oxidations has led to suggestions that these tissues possess a terminal oxidase other than cytochrome oxidase. However Chance and Hackett (9) have criticized these suggestions on the basis that the results can equally well be explained on an excess-oxidase theory. The excess-oxidase theory assumed that a very small percentage of the total cytochrome oxidase is capable of maintaining electron transport. Assuming that the cytochrome oxidase of mitochondria is not compartmentally divided with respect to its reaction with cyanide, the excess-oxidase theory does not account for the observation (32) that in the presence of mM KCN the residual succinoxidase activity was 75 times greater than the residual cytochrome oxidase activity. A similar effect, though not as striking, is shown in figure 10, where a mixture, PA, was used to assay cytochrome oxidase. Since PA was oxidized much faster than succinate, it was assumed that an inhibition of the PA rate of oxidation by cyanide reflected the maximum activity of the remaining uninhibited cytochrome oxidase. However, in the presence of mM KCN succinate was oxidized twice as fast as PA. Furthermore, addition of succinate to the cyanide-inhibited PA system stimulated oxidation to the level of the cyanide-inhibited succinate system. Reversing this procedure showed that PA had little effect on the cyanide-inhibited succinate oxidation. These effects, though small, could be reproduced with malate and were observed with all of the sweet potato preparations that were tested. However, the white-potato mitochondria did not show this type of result on any occasion. In this case the residual rates of oxidation were very small for both succinate and PA.

The oxidation of succinate (or malate) in the presence of mM KCN was sensitive to HOQNO whereas that of PA was insensitive.

Discussion

The method used for isolating mitochondria as described here has proved successful in that tightly coupled and controlled respiration was observed with particulates isolated from difficult tissues. Although the general methodology is well known (19) rigid

control of critical factors must be maintained for optimal activity and respiratory control. In this work it was found that changes in temperature and pH had marked effects on the final preparation. The use of a medium with suitable density facilitated separation of the mitochondria. Lieberman (28) has made a detailed study on the effect of pH of isolation on apple mitochondria. Active preparations were obtained only from alkaline media. However, under such conditions only the more stable mitochondrial components would remain active. Thus, although maximum rates of oxidation were operating, phosphorylation could not be detected, indicating some modification of the particles. Preparation of sweet potato mitochondria as described by Hackett et al. (22) yielded preparations which showed no respiratory control and a strong stimulative response to added cytochrome c, while those of Baker and Lieberman (3), exhibited neither respiratory control nor response to added cytochrome c. It is suggested that these different responses are a result of the isolation technique. Maintaining the mitochondria in a solution containing EDTA may result in removal of metals catalytic to respiratory processes (33) and the continued presence of tris in the media leads to the extraction of cytochrome c (W. Bonner, unpublished data).

It has been demonstrated that maximal substrate oxidation does not occur unless a suitable phosphate acceptor is present. Moreover, on the exhaustion of phosphate acceptor, the respiration rate decreases indicating respiratory control and therefore tight coupling (cf. ADP/O ratios). A mere stimulation of plant mitochondrial substrate oxidation following ADP addition cannot be taken to represent respiratory control (22, 34) unless it is established that ADP is rate limiting. The absence of respiratory control does not reflect on the phosphorylation capacity, as it may be due to the presence of adenosine triphosphatases. Evidence for this was shown by the use of oligomycin which inhibits phosphorylation without uncoupling (26). Dinitrophenol was capable of relieving this inhibition of oxidation.

It has been demonstrated that DNP can stimulate substrate oxidation. However, the level of DNP necessary for a maximum oxidation rate (40–50 μM) is much lower than that reported for a high degree of uncoupling. Thus DNP inhibits at effective uncoupling concentrations, an observation that explains why reports of DNP stimulated oxidation in plant mitochondria are rare in the literature (19). It has also been shown that factors other than phosphorylation may be limiting oxidation and under such circumstances DNP does not stimulate oxidation, even though it may uncouple. A stimulation of state 3 with DNP cannot be expected unless phosphorylation is rate limiting, a condition not yet demonstrated in plant mitochondria.

The ability to measure initial rates of substrate oxidation has shown that freshly isolated plant mitochondria are often severely inhibited; ATP is required to relieve this inhibition. The inhibition was shown to develop during succinate oxidation in the presence of DNP or during malate oxidation prior to succinate addition. Amytal prevented the development of this inhibition, a result suggesting the inhibiting agent to be oxaloacetate. Similar effects have been demonstrated in rat liver mitochondria (13). ATP may either dissociate the oxaloacetate-succinate dehydrogenase complex or remove oxaloacetate completely by the phosphoenolpyruvate carboxykinase reaction as suggested by Pardee and Potter (30) and Chappell (13). It has been shown (29) that phosphoenolpyruvate carboxykinase is widely distributed in plant tissues and that the plant enzyme is specific for ATP. This latter observation could explain why nucleotides other than ATP were ineffective in removing oxaloacetate inhibition.

In rat liver homogenates the oxaloacetate inhibition of succinate oxidation is removed by incubation with glutamate (25), no evidence of such transaminations were observed in the sweet potato and white potato mitochondria. One of us (W. Bonner) has found the glutamate oxaloacetate transamination in wheat and barley root mitochondria and Throneberry's (35) observations on the beneficial effects of glutamate are probably due to the same reaction. It is interesting that Throneberry (35) observed a glutamate inhibition of isocitrate oxidation. A mechanism of this latter inhibition has been postulated (13).

The observation that succinate oxidation can be inhibited through conversion to oxaloacetate explains the DPN inhibition of succinate oxidation in the experiments of Throneberry (35) and Beevers and Walker (4). In mitochondria that are not reasonably intact (lack respiratory control), the presence of DPN would stimulate malate oxidation, thus allowing oxaloacetate accumulation.

The use of amytal has yielded conflicting results in different laboratories (19). Here it has been shown that amytal inhibits malate oxidation under phosphorylating conditions, there being no marked inhibition under nonphosphorylating conditions. The evidence indicates that amytal is most effective in inhibiting low rates of malate oxidation, e.g., perhaps that resulting from succinate oxidation. It is suggestive that endogenously formed malate is preferentially oxidized via an amytal sensitive phosphorylating pathway, while added malate is oxidized through an amytal resistant nonphosphorylating pathway. Indeed, Chance (7) has shown differences in the effects of amytal on rat liver mitochondria under phosphorylating and nonphosphorylating conditions. A clarification of the effects of amytal on plant mitochondria is needed.

The relatively small inhibitions of organic acid oxidation observed with mM cyanide cannot be taken to indicate cyanide-insensitive respiration. However the fact that this same concentration of cyanide has a marked effect on the oxidation of a cytochrome-oxidase substrate shows that cyanide remains an ef-

fective cytochrome-oxidase inhibitor. When it is observed that in the presence of mM cyanide the residual rate of organic acid oxidation is far in excess of the residual rate of cytochrome-oxidase activity (32 and figure 2) some conclusions can be drawn. If it is assumed that the rate of PA oxidation in the presence of cyanide represents maximal cytochrome-oxidase activity, a stimulation of oxidation by succinate or malate reflects another pathway of oxidation. Moreover, this pathway is sensitive to HOQNO. Consideration of figure 10 shows that cytochrome oxidase can be inhibited to the extent of 74 % without affecting succinate oxidation. Such calculations show that the excess hypothesis cannot be ignored in those cases where little or no apparent inhibition is observed with cyanide.

The investigations on cyanide described in this paper clearly show an alternate pathway to O_2 in some plant tissues, they do not allow any deductions concerning the nature of this alternate pathway.

Summary

Procedures are described for preparing mitochondria exhibiting the property of respiratory control from both sweet potato and white potato tissues. The oxidative properties of these particles were studied polarographically. It was found that the succinate oxidation of freshly isolated particles could be severely inhibited and that this inhibition was not directly related to oxidative prosphorylation although ATP or some high-energy intermediate was needed to overcome the inhibition.

It was concluded that the inhibition of succinate oxidation was due to oxaloacetate and that the ATP dissociated the oxaloacetate-succinatedehydrogenase complex.

A study was made of some respiratory chain inhibitors, including oligomycin, amytal, antimycin A, 2-in-heptyl-4-hydroxyquinoline-N-oxide, and cyanide. The dinitrophenol-oligomycin interactions were also studied. Some evidence is presented to suggest that the sweet potato mitochondria possess in addition to cytochrome oxidase an alternate oxidase.

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