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RESPIRATORY PARTICLES FROM CACTUS PHYLLOCLADES

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Abstract—Respiratory particles, presumably contaminated with chloroplast fragments, isolated from cactus phylloclades, gave tests for the enzymes of the tricarboxylic acid cycle and respiratory chain complexes, without special treatment for membrane disruption. However, detergent treatment resulted in a marked stimulation of many of the activities. The (untreated) particles were capable not only of oxidizing the tricarboxylic acid cycle acids, but also of satisfactorily coupling their oxidation with phosphorylation. Results obtained with inhibitors lent support to the occurrence of enzymes of the tricarboxylic acid cycle and the electron transfer chain. As the tissue aged, the respiratory particles showed a lowered ability to oxidize succinate and, more so, to effect esterification of phosphate; there was also a lowered activity of the enzyme complexes of the respiratory chain.

INTRODUCTION

THERE is strong evidence that the tricarboxylic acid cycle (TCA cycle) is operative in succulents.[1,2] Nevertheless, apart from the reports by Brandon[3,4] on the isolation from the leaves of *Bryophyllum tubiflorum* of particles with malate dehydrogenase activity and the ability to oxidize succinate and malate with phosphorylation, and the report by Mukerji and Ting[5] on malate dehydrogenase activity in the mitochondrial fraction from *Opuntia ficus-indica*, no detailed study exists on the respiratory activity of mitochondrial preparations in succulent plant tissues. An earlier report from this laboratory dealt with the cytochrome oxidase of cactus.[6] The present communication deals with the isolation from cactus phylloclades of particles, made up of mitochondria presumably contaminated with chloroplast fragments, with apparently a full complement of the TCA cycle enzymes and the enzymes of the electron transfer pathway and functionally active in oxidative phosphorylation.

RESULTS

Enzymic Activity in Intact Mitochondrial Preparations

The activities of all the TCA cycle enzymes and components of the respiratory chain tested were detectable in the untreated mitochondrial preparations (Table 1).

Of the TCA cycle enzymes, fumarate hydratase activity was the highest, followed closely by succinate dehydrogenase and aconitate hydratase. Malate dehydrogenase activity was also high, but represented only one-half the activity of fumarate hydratase. Isocitrate dehydrogenase (NADP-linked and NAD-linked) and oxoglutarate dehydrogenase constituted a second group of enzymes, with distinctly lower specific activity.

[1] S. L. RANSON, *Plant Biochemistry*, p. 493, Academic Press, London and New York (1965).
[2] S. L. RANSON and M. THOMAS, *Ann. Rev. Plant Physiol.* **11**, 81 (1960).
[3] P. C. BRANDON, *Proc. Koninkl. Ned. Akad. Wetenschap. Ser C, Amsterdam* **66**, 406 (1963).
[4] P. C. BRANDON, *Plant Physiol.* **42**, 977 (1967).
[5] S. K. MUKERJI and I. P. TING, *Phytochem.* 7, 903 (1968).
[6] A. A. KHAN, C. P. TEWARI, P. S. KRISHNAN and G. G. SANWAL, *Plant Physiol.* **43**, 1461 (1968).

TABLE 1. ENZYMIC ACTIVITIES OF THE MITOCHONDRIA

Enzymes	Specific activity (units/mg protein)	
	Without detergent	With detergent
Tricarboxylic acid cycle		
Aconitate hydratase	0·300	0·900
Isocitrate dehydrogenase		
(NADP-linked)	0·071	0·137
(NAD-linked)	0·034	0·071
Oxoglutarate dehydrogenase	0·067	
Succinate dehydrogenase	0·310	0·620
Fumarate hydratase	0·413	1·667
Malate dehydrogenase	0·207	0·551
Respiratory chain		
NADH-cytochrome *c* reductase	0·041	
Succinate-cytochrome *c* reductase	0·051	
Cytochrome oxidase*	0·136	0·231
Diaphorase	0·076	
NADH oxidase	0·034	0·071
Succinate oxidase	0·020	

The mitochondrial preparations were isolated in sucrose–mannitol medium. A different batch was used for studies on respiratory chain enzymes.

* Activities were estimated with 15 μM ferrocytochrome *c*; the activity at infinite concentration of ferrocytochrome *c* was 0·57 units/mg protein, without detergent treatment.

Among component activities of the respiratory chain, cytochrome oxidase activity was markedly higher than others. Diaphorase activity was high, but NADH cytochrome *c* reductase and NADH oxidase activities were low. Succinate-cytochrome *c* reductase had fairly high activity, but succinate oxidase activity was very low.

Enzymic Activity in Detergent-treated Mitochondrial Preparations

Oxoglutarate dehydrogenase was not assayed, but there was an increase in the specific activity of all other enzymes. Fumarate hydratase, aconitate hydratase, succinate dehydrogenase and malate dehydrogenase again formed a distinct group of enzymes with high activity. In comparison, isocitrate dehydrogenase (both NAD and NADP-linked) had low activity. The treated preparations retained the same relative order of gradation of specific activities as the control preparations, with the difference that whereas aconitate hydratase and succinate dehydrogenase had the same activity in the untreated preparation, the activity of the former was 50 per cent higher in the treated preparation. Of the respiratory chain enzymes, cytochrome oxidase and NADH oxidase were activated to an equal extent. NADH cytochrome *c* reductase and succinate cytochrome *c* reductase were greatly inhibited following detergent action.

Phosphate Esterification Coupled to the Oxidation of TCA Cycle Intermediates

The mitochondrial preparations from cactus phylloclades isolated in the medium of Pierpoint[7] were tested for the oxidation of TCA cycle intermediates (40 μmoles of each) and for the coupled phosphorylation.

[7] W. S. PIERPOINT, *Biochem. J.* **71**, 518 (1959).

The P:O ratio was highest for malate and for oxoglutarate (Table 2). The ratio for succinate was the closest approximation to the generally accepted limiting values. In isolated experiments the P:O ratios approached the presumed maximum for all substrates other than oxoglutarate.

TABLE 2. OXYGEN UPTAKE AND COUPLED PHOSPHORYLATION DURING OXIDATION OF THE TRICARBOXYLIC ACID CYCLE INTERMEDIATES

Substrate	O_2 uptake (μatom/reaction flask)	P_i esterified (μatom/reaction flask)	P:O
Citrate	2·50 ± 0·8	5·22 ± 1·4	2·09 ± 0·7
Oxoglutarate	3·54 ± 1·2	7·85 ± 2·9	2·22 ± 0·2
Succinate	4·25 ± 0·6	7·65 ± 1·4	1·80 ± 0·3
Fumarate	2·63 ± 0·6	4·97 ± 0·3	1·89 ± 0·5
Malate	2·39 ± 0·4	5·40 ± 1·0	2·30 ± 0·4
Pyruvate*	2·10	2·64	1·26

The reaction system contained in the main compartment of each flask: 400 μmoles sucrose, 10 μmoles phosphate buffer, pH 7·2, 10 μmoles $MgSO_4$, 0·1 μmole $MnSO_4$, 15 μmoles sodium fluoride, 2 μmoles ATP, 0·018 μmole cytochrome *c*, 0·1 μmole TPP, 0·2 μmole NAD, 0·1 μmole NADP, mitochondrial preparation (equivalent to 2 g fresh weight of tissue) and water to 2·55 ml. The side arm contained in 0·45 ml: 20 μmoles glucose, 40 μmoles substrate acid adjusted to pH 7·2, and 1 mg hexokinase (Sigma, Type II). The values reported are the means ±SD of six observations.

* In addition to other components, 0·5 μmole succinate and 0·01 μmole of CoA were added. The reaction flask contained mitochondria from 5-g fresh tissue. The data were not corrected for oxidation of added succinate.

Incubation with 40 μmoles pyruvate and 0·01 μmole of CoA did not result in any measurable oxygen uptake even when the amount of mitochondria used was increased 2·5-fold. On supplementation of system with 0·5 μmole succinate, oxidation and phosphate esterification occurred.

Factors Affecting Oxidation and Phosphorylation of Succinate

Oxidative phosphorylation was assayed in a reaction system which contained a variety of cofactors and fluoride.[8] The need or otherwise for some of the individual factors was tested in separate experiments with succinate as substrate, employing mitochondrial preparations isolated in the media of Wiskich and Bonner[9] and of Pierpoint.[7]

Cytochrome c. Cytochrome *c* promoted the oxidation by 30 to 40 per cent with mitochondria isolated in the medium of Wiskich and Bonner,[9] but about 3-fold in corresponding preparations in the medium of Pierpoint.[7] The preparations in the former medium also exhibited greater efficiency of coupled phosphorylation.

ATP. ATP had practically no effect on the oxidation in mitochondria. In its absence, phosphate esterification was practically completely abolished with mitochondria isolated in the medium of Wiskich and Bonner;[9] with mitochondria prepared in Pierpoint's medium, it was reduced to less than a seventh.

In a separate experiment, where 2 μmoles of ADP was used in place of ATP, the esterification of orthophosphate by a mitochondrial preparation isolated in the sucrose–mannitol medium was six times as great as that in the absence of ADP. The oxidation in the presence of ADP showed 44 per cent stimulation, viz. a respiratory control ratio of 1·44.

[8] W. S. PIERPOINT, *Biochem. J.* **75**, 504 (1960).
[9] J. J. WISKICH and W. D. BONNER, JR., *Plant Physiol.* **38**, 594 (1963).

Hexokinase. There was a drop (22 to 37 per cent) in the rate of oxidation of succinate when hexokinase was withheld. The esterification of phosphate was reduced to very low levels (16 to 24 per cent).

Sodium fluoride. The oxidation with a mitochondrial preparation isolated in Wiskich–Bonner's medium was not affected when fluoride was withheld, but there was a 22 per cent drop with the preparation isolated in Pierpoint's medium. The phosphorylation was practically completely suppressed in either case.

Other components. NAD, thiamine pyrophosphate and $MgSO_4$ had negligible effects on the oxidation of succinate. The phosphorylation was not tested in the absence of these agents.

Effect of Inhibitors and Uncoupling Agents on Mitochondrial Activity (*See* Table 3)

TABLE 3. EFFECT OF INHIBITORS AND DINITROPHENOL ON OXIDATION AND PHOSPHORYLATION BY CACTUS MITOCHONDRIA

Substrate	O_2 uptake (μatom/reaction flask)	P_i esterified (μatom/reaction flask)	P:O	O_2 uptake (μatom/reaction flask)	P_i esterified (μatom/reaction flask)	P:O
Experiment I						
		Control			Cyanide, 5×10^{-3}M	
Oxoglutarate	2·10	5·04	2·40	1·58 (24·8)	0 (100)	0
Succinate	3·95	7·56	1·90	2·67 (32·6)	0 (100)	0
Malate	2·13	5·40	2·53	1·64 (23·0)	0 (100)	0
Experiment II						
		Control			Azide, 1×10^{-3}M	
Citrate	2·25	5·04	2·24	1·76 (22·2)	1·08 (78·5)	0·61
Oxoglutarate	3·83	7·56	1·97	3·21 (16·2)	1·08 (85·7)	0·33
Succinate	3·90	6·12	1·57	3·00 (23·0)	1·08 (82·3)	0·36
Fumarate	2·32	5·04	2·17	1·70 (22·4)	1·08 (78·5)	0·63
Malate	2·39	5·40	2·26	1·73 (32·0)	0 (100)	0
Experiment III						
		Control			Malonate, $1{\cdot}5 \times 10^{-5}$M	
Succinate	3·95	7·56	1·90	2·53 (36·3)	4·32 (43·9)	1·70
Experiment IV						
		Control			Dinitrophenol, 1×10^{-4}M	
Citrate	2·01	3·95	1·97	2·22 +(9·5)	1·44 (63·4)	0·65
Oxoglutarate	2·79	5·04	1·81	2·21 (20·3)	0·72 (85·7)	0·33
Succinate	4·20	7·20	1·71	4·42 +(5·0)	2·16 (70·0)	0·49
Fumarate	2·44	3·96	1·62	2·31 (5·3)	1·44 (63·9)	0·62
Malate	2·16	3·96	1·83	2·09 (3·2)	1·80 (54·5)	0·85

Each reaction flask contained mitochondria, isolated in the medium of Pierpoint, derived from 2 g tissue. The assay system was the same as described in Table 2. In experiments with cyanide, the central well of the flask contained $Ca(CN)_2$–$Ca(OH)_2$. Figures in parentheses indicate percentage inhibition. A positive sign prefixed indicates stimulation.

Cyanide. The oxidation of succinate was inhibited 33 per cent and of malate and oxoglutarate 25 per cent each by 5×10^{-3} M cyanide. Phosphate esterification was completely abolished in every case. In a separate experiment citrate oxidation was inhibited 10 per cent and the esterification of phosphate was completely abolished.

Azide (1×10^{-3} M) resembled cyanide in inhibiting phosphate esterification to a greater extent than oxidation. The inhibition of the former was 80 to 85 per cent with succinate, citrate, oxoglutarate and fumarate and was complete with malate. The oxidation of the various substrates was inhibited by 15 to 30 per cent.

Malonate ($1{\cdot}5 \times 10^{-5}$ M) inhibited, nearly to the same extent, the oxidation of succinate and the attendant phosphate esterification, so that the P:O ratio was not markedly affected.

Dinitrophenol (1×10^{-4} M) inhibited by 55 to 86 per cent the phosphate esterification occurring during the oxidation of organic acids. It had a negligible effect on the oxidation of fumarate, malate and succinate; but inhibited oxoglutarate oxidation by 20 per cent and stimulated citrate oxidation by 10 per cent.

Antimycin A. Antimycin A inhibited NADH oxidase, NAD cytochrome *c* reductase and succinate cytochrome *c* reductase. During the assay of NADH cytochrome *c* reductase and NADH oxidase, antimycin A—5 and 9 μg per ml in the former and 3 μg per ml in the latter system—inhibited the activities by 50 per cent in the first minute of observation; on continuing the incubation to 2·5 min, no further inhibition occurred. Succinate cytochrome *c* reductase activity was more susceptible to antimycin A. During the first min, 3 μg per ml antimycin A inhibited the activity by 75 per cent and 5 μg per ml led to complete inhibition. Within 2 min, complete inhibition occurred also with the lower level of antibiotic.

Effect of Ageing of Tissue on the Activity of Mitochondria

Tender phylloclades (about 2 weeks of growth) and the parent phylloclades (5–6 months of growth) were simultaneously worked up for the mitochondrial fraction, using the medium of Wiskich and Bonner.[9] Analyses of the activities of respiratory chain enzyme complexes and for the oxidation of succinate are reported in Tables 4 and 5.

TABLE 4. EFFECT OF AGEING OF TISSUE ON OXIDATION OF SUCCINATE AND ON PHOSPHATE ESTERIFICATION BY ISOLATED MITOCHONDRIA

	Protein (mg/g fr. wt.)	O_2 uptake, μatom/ mg protein/	g fr. wt.	P_i esterified, μatom/ mg protein/	g fr. wt.	P:O
Experiment I						
Tender	0·98	1·35	1·33	2·10	2·06	1·56
Mature	0·84	0·58	0·49	0·28	0·27	0·50
Decrease (%)	14·3	57·0	63·2	86·8	86·8	68·0
Experiment II						
Tender	0·98	1·46	1·43	2·10	2·05	1·44
Mature	0·77	0·58	0·46	0·30	0·17	0·51
Decrease (%)	21·4	59·5	67·8	85·7	91·3	64·6

The mitochondrial fraction isolated from mature phylloclades had 14 to 21 per cent less protein per g fresh weight of tissue than that from the tender tissue. Maturation of tissue was attended with a slowing down of the oxidative reactions. The activities of NADH cytochrome

c reductase, succinate cytochrome *c* reductase, diaphorase and cytochrome oxidase were lowered in the range of 22 to 58 per cent on the basis of protein and 33 to 67 per cent per g equivalent of fresh tissue. There was a marked reduction in the rate of oxidation of succinate and still more marked reduction in phosphate esterification by mitochondria from mature tissue, resulting in a 65 to 68 per cent reduction of the P:O ratio.

TABLE 5. EFFECT OF AGEING OF TISSUE ON THE ACTIVITIES OF MITOCHONDRIAL RESPIRATORY CHAIN ENZYMES

	NADH-cytochrome *c* reductase		Succinate-cytochrome *c* reductase		Diaphorase		Cytochrome oxidase*	
	per mg protein (units)	per g fr. wt. (units)	per mg protein (units)	per g fr. wt. (units)	per mg protein (units)	per g fr. wt. (units)	per mg protein (units)	per g fr. wt. (units)
Experiment I								
Tender	0·055	0·054	0·026	0·027	0·041	0·040	0·055	0·054
Mature	0·043	0·036	0·011	0·009	0·026	0·022	0·037	0·031
Decrease (%)	21·8	33·3	57·7	66·7	45·0	32·7	32·7	43·0
Experiment II								
Tender	0·037	0·036	0·027	0·027	0·032	0·031	0·064	0·063
Mature	0·023	0·018	0·012	0·009	0·023	0·016	0·041	0·031
Decrease (%)	37·9	50·0	55·6	66·7	28·1	48·4	35·9	50·5

* Concentration of reduced cytochrome *c* employed was 15 μM.

DISCUSSION

Special difficulties were encountered in the isolation of the mitochondrial particles from cactus tissue due to the highly acidic cell sap, the low concentration of protein, the high concentration of mucilage, the occurrence of both starch grains and chloroplasts and the presence of a tough cuticle. By a close adherence to the procedure described, it was possible to obtain consistently active preparations of the particles with reproducible activity.

The various intermediates of the TCA cycle were oxidized efficiently with coupled phosphorylation. In this respect, the cactus particles differed from the mitochondria from mung bean, potato, sweet potato and cauliflower[10] and resembled those from the tomato fruit.[11] The absence of an initial inhibition in the oxidations of citrate, succinate and fumarate was in contrast to the lag observed by Zelitch and Barber,[12] who attributed the lag to a likely limiting concentration of fumarate—and aconitate hydratases and by Wiskich and Bonner,[9] who attributed the initial inhibition of succinate oxidation to the presence of oxaloacetate. P:O ratios for various intermediates obtained with cactus preparations were comparable with those of the most active preparations from other plants.[13–15]

[10] W. D. BONNER, JR., *Plant Biochemistry*, p. 89, Academic Press, London and New York (1965).
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[12] I. ZELITCH and G. A. BARBER, *Plant Physiol.* **35**, 205 (1960).
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Results obtained with the conventional inhibitors lent support to the occurrence of enzymes of the tricarboxylic acid cycle and an organized electron transfer chain. The pattern of inhibitor-induced response on the P:O ratios is similar to that of other plant preparations. Dinitrophenol did not stimulate oxygen uptake. In this respect cactus particles resembled the mitochondria isolated from sweet potato[9,16] and green tomato fruits[17] but differed from that of etiolated lupin seedlings.[18] Like many other preparations,[19-22] the oxygen uptake of cactus mitochondria was partially insensitive to inhibition by cyanide. Although the P:O ratios were satisfactory, the low value for acceptor ratio (the ratio of oxygen uptake in the presence and absence of phosphate acceptor) argued against tightness of coupling.

The high activity of cytochrome oxidase suggests that the major part, if not the whole, of tissue respiration is mediated through cytochrome oxidase. The specific activity of aconitate hydratase in (untreated) cactus mitochondrial preparations was consistently higher than that of many other plant preparations.[23] The fumarate hydratase activity of the least active sample of the (untreated) mitochondrial preparation isolated from cactus was comparable with that of the highest recorded activity for mitochondria, namely that of tobacco leaf,[24] but most of the preparations, such as the one reported in Table 1, had higher activity. The high activities of these two enzymes, especially as determined in the detergent-treated mitochondrial preparations, may probably be associated with the Crassulacean type of metabolism of the plant. The inability of Bacon *et al.*[23] to demonstrate any significant aconitate hydratase activity in extracts of green leaves of *Bryophyllum crenatum*, *Crassula perforata*, *Rochea coccinea* and *Sedum prealtum* can be explained only in part by the occurrence of an enzyme inhibitor in these tissues.

The maturation of cactus phylloclades was found to be associated with a marked lowering of activity of respiratory chain complexes and the ability to oxidize succinate. The effect of ageing of the tissue was specially marked on the P:O ratio, suggesting that the phosphate-trapping mechanism was more affected than the respiratory pathway. The observed changes could be explained by assuming that the lowered mitochondrial activity is characteristic of mature tissue, or that mitochondrial isolation from mature tissue is associated with damage to structure,[25] or inhibition by toxic agents. The experimental findings on the biochemical changes in the activity of cactus mitochondria from young and mature tissue, thus, resemble the pattern of changes noted by Geronimo and Beevers;[25] the findings agreed with the observations of Zelitch and Barber[12] with spinach leaves as far as the reduction in oxidation was concerned, but differed in that phosphate esterification was more sensitive than oxygen uptake.

EXPERIMENTAL

Tissue

The cactus used was *Nopalea dejecta* Salm Dyck., grown in garden soil under natural conditions of moisture, temperature and sunlight. Unless otherwise specified, tender phylloclades were used within 2 weeks of

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[18] E. E. Conn and L. C. T. Young, *J. Biol. Chem.* **226**, 23 (1957).
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sprouting on well-established bushes. The harvesting was between 8 to 11 a.m. The influence of ageing was studied by comparison of the tender phylloclades with the thickened parent phylloclades.

Isolation of Cell Fractions

(*a*) *Sucrose medium with supplements.* The medium of homogenization, based on that of Pierpoint, consisted of 0·4 M sucrose containing 0·1 M phosphate buffer, 0·02 M sodium citrate and 5 mM EDTA adjusted to pH 7·8. The washed tissue was ground in the cold in a Waring Blendor with 2·5 to 3 vol. of the chilled medium, at low speed for 15 sec followed by 10 sec at full speed. After squeezing through two layers of muslin into an iced container, the homogenate (pH 7·0–7·2) was centrifuged to give particulate fractions at 200 *g* (5 min, washed once), the debris and starch fraction, 1600 *g* (10 min, washed once), the chloroplast fraction, 15,000 *g* (30 min, washed twice), the mitochondrial fraction and the supernatant fraction. Sediments were washed with 0·4 M sucrose in 0·05 M phosphate buffer, pH 7·2; the same medium was used in the final dispersion of the particles using a Potter–Elvehjem homogenizer. 1 ml of the suspension contained material derived from 2 g fresh phylloclades.

(*b*) *Mannitol–sucrose medium with supplements after Wiskich and Bonner.*[9] The dispersion medium was made up of 0·32 M mannitol, 0·25 M sucrose, 5 mM EDTA and 0·1 M Tris, pH adjusted to 7·8 with HCl, giving homogenates at pH 7·0 to 7·2. A tissue:medium ratio of 1:4 or 1:5 (w/v) was best suited for homogenization. The fractions sedimented under the centrifugal fields mentioned above were washed with 0·32 M mannitol in 0·25 M sucrose, and the particles were finally suspended in 0·5 M mannitol.

Chemical Composition of Mitochondrial Fraction

Protein. The protein content of the fraction, 0·7–1·0 mg per g fresh weight equivalent of cactus tissue, isolated in sucrose–mannitol medium was slightly, but consistently, higher than that isolated in sucrose-medium.

Starch. The mitochondrial fraction contained 0·06–0·09 mg per g equivalent of cactus tissue, representing 2 to 3 per cent of the total starch in tissue. The content was slightly lower in the isolate in sucrose–mannitol medium.

Chlorophyll. The mitochondrial fractions isolated in the medium of Wiskich and Bonner[9] contained 0·30 to 0·38 mg chlorophyll per g equivalent of tissue, representing a recovery of 33 to 39 per cent of chlorophyll in the homogenate.

Nucleic acid. The mitochondrial fraction isolated in the medium of Wiskich and Bonner[9] contained 2·2 μg RNA phosphorus and 0·11 μg DNA phosphorus per g equivalent of cactus tissue, representing 8 per cent and 1 per cent of the total content in homogenate.

Enzyme Activity Determination

In the assay of succinate dehydrogenase in the mitochondrial fraction, the absorption measurements were made after deproteinization of the assay system with trichloroacetic acid. All the other enzymic activities in the mitochondrial fraction were determined by changes in the absorption during the course of enzyme action. Units of enzyme activity are μmoles substrate transformed per min under the assay conditions at 30°. The specific activity of an enzyme is units of activity per mg protein.

Aconitate hydratase was assayed according to Racker.[26] Isocitrate dehydrogenase (NADP-linked) was assayed according to Scott *et al.*[27] after Davies,[28] oxoglutarate dehydrogenase according to Garland;[29] succinate dehydrogenase after Slater and Bonner;[30] fumarate dehydrogenase according to Racker;[26] malate dehydrogenase after Ochoa;[31] NADH cytochrome *c* reductase, succinate cytochrome *c* reductase and NADH oxidase according to Hackett *et al.*;[32] diaphorase according to Tamaoki *et al.*;[33] cytochrome oxidase according to Cooperstein and Lazarow[34] in the spectrophotometric assays and according to Lieberman[35] in manometric assays and succinate oxidase according to Pierpoint.[36] Cyanide (3 mM) was added in assays of isocitrate (NAD and NADP-linked), malate and oxoglutarate dehydrogenases to prevent the reoxidation through the respiratory chain. Data for cytochrome oxidase[6] and NADH cytochrome *c* reductase activities were analysed for the first-order reaction kinetics; all the other reactions followed the zero-order kinetics with respect to substrate.

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Oxidative phosphorylation was measured according to Pierpoint;[8] coenzyme A was omitted unless otherwise specified. All studies were carried out on preparations with 1 hr of isolation.

Detergent Treatment

Enzyme activities were determined in untreated mitochondrial fraction and in detergent-treated fractions.[37] NAD-linked isocitrate dehydrogenase was assayed in the presence of 0·02 per cent (w/v) sodium deoxycholate added during the estimation. Triton X-100 was used in the assay of other enzymes. For aconitate hydratase and malate dehydrogenase there was a period of preincubation, the former for 5 min with 0·04 per cent (v/v) detergent and the latter for 10 min with 0·4 per cent (v/v) detergent. The assay system for NADP-linked isocitrate dehydrogenase contained 0·4 per cent (v/v), that for succinate dehydrogenase, fumarate hydratase and cytochrome oxidase 0·02 per cent (v/v) and NADH oxidase 0·4 per cent (v/v) detergent, added during estimation.

Other Estimations

Chlorophyll estimation in homogenates and subcellular fractions was according to Arnon,[38] orthophosphate according to Lowry and Lopez[39] in the determination of phosphate esterification, and according to Bartlett[40] in the estimation of nucleic acid phosphorus, starch according to Pucher *et al.*[41] and protein according to Lowry *et al.*[42] Phosphorus fractionation for nucleic acid was according to Schmidt and Thannhauser[43] and Schneider.[44] Cyanide used as inhibitor during oxidative phosphorylation was prepared according to Robbie.[45] Cytochrome *c* was reduced by dithionite according to Potter.[46]

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