STUDIES IN THE METABOLISM OF PLANT CELLS

X. RESPIRATORY ACTIVITY AND IONIC RELATIONS OF PLANT MITOCHONDRIA

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[*Manuscript received November* 26, 1954]

Summary

The respiratory activity and ionic balance of mitochondria isolated from carrot and beet tissues by differential centrifugation have been studied. The oxygen uptake of the mitochondria with different substrates was investigated. The mitochondria hold both cations and anions in concentrations greater than those in the supernatant. Experiments on the time of adjustment to a changed concentration of chloride in the supernatant solution have been used to calculate the diffusion constant of salt in the particle. On the assumption that most of the resistance to diffusion is in the surface membrane (thickness about 200 Å), the apparent diffusion constant of chloride in the membrane was shown to be of the order of 10^{-13} cm^2/sec. This agrees with that found for heart muscle sarcosomes under similar conditions and is of the order expected in a lipo-protein membrane. The concentrations of mobile cations (Na^+ and K^+) in the mitochondria are considerably greater than those in the supernatant. It is suggested that the internal concentrations are largely due to a Donnan equilibrium based on the immobile anions of the particle. Since no simple Donnan equilibrium will account for the simultaneous concentration of both mobile cations and mobile anions, it is suggested that the mobile anions might be accumulated by an accumulatory mechanism. The anion concentration difference between the inside and outside of the particles is of the order of magnitude to be expected if the electron carrier of respiration were acting as the anion carrier of accumulation. The results are therefore not inconsistent with the earlier hypotheses for the interdependence of the two processes. The results support the hypothesis that mitochondria are probably involved in electrolyte accumulation in plant cells and in secretion in animal cells such as those of the gastric mucosa.

I. INTRODUCTION

The participation of a cytochrome oxidase system in the salt respiration of cut plant tissue has been demonstrated (Weeks and Robertson 1950). It has been suggested that salt accumulation depends on salt respiration (Lundegårdh 1940; Robertson and Turner 1944) and that the mechanism might be associated with the cytochrome system. Robertson and Wilkins (1948) showed that the quantitative relation between salt respiration and accumulation is consistent with the hypothesis that the separation of hydrogen ions and electrons at the cytochrome system is part of the mechanism.

If the cytochrome system is part of the mechanism of salt accumulation it is desirable to know where this system of enzymes is located in the cell. By

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analogy with animal enzymes it was suggested (Robertson 1951) that since the cytochrome probably occurred on the mitochondrial particles, the mitochondria are implicated in the processes of salt respiration and accumulation. Since then Stafford (1951) and Millerd (1951) have established that plant cytochrome oxidase occurs in intracellular particles, of the size of mitochondria. Further, Millerd *et al.* (1951) showed that, as with animal cells, other respiratory enzymes occur on these particles. These results have been confirmed by Davies (1953), Laties (1953*a*, 1953*b*), and Brummond and Burris (1954).

The purpose of this paper is to record investigations on the respiratory activity and ionic relations of intracellular particles from carrot and beet tissue. This work was done in conjunction with a study of the morphology of the particles by phase contrast and electron microscopy (Farrant, Robertson, and Wilkins 1953, and unpublished data). The study of the ionic relations of the plant cell particles is essential to the consideration of their possible role in accumulation. Accumulation of cations in animal mitochondria has been demonstrated by Bartley and Davies (1952, 1954), Macfarlane and Spencer (1953), and Stanbury and Mudge (1953); Bartley and Davies also demonstrated that phosphate and organic acid anions were concentrated in mitochondria.

II. Material

The xylem parenchyma of carrot root (*Daucus carota* L.) and the heterogeneous tissues of the beet root (*Beta vulgaris* L.) were used. These tissues were chosen because they have been used for respiration and accumulation studies in earlier work. Prior to blending, the tissue was prepared in the cold by cutting into cubes of sides approximately 0·5 cm. Cytochrome *c* was prepared by the method of Keilin and Hartree as quoted by Umbreit, Burris, and Stauffer (1949). The final dialysis was made against distilled water instead of sodium chloride solution.

III. Methods

The particles were extracted in the cold by disintegrating the tissue, separating the larger constituents by squeezing through muslin, and centrifuging at different speeds to separate the particles. As the tissues consist largely of mature, highly vacuolated cells, a large volume of tissue was necessary to obtain a relatively small number of mitochondria. Facilities for preparing the particles were improved as the work progressed.

(*a*) *Preparation of Particles*

The tissue was disintegrated in a Waring Blendor for 30 sec. Approximately equal volumes of tissue and blending medium were used in the blendor bowl. Sucrose (15 per cent.) was found to be the most satisfactory blending medium as judged by oxygen uptake. In both beet and carrot tissues the homogenate came to a pH of approximately 6; neutralizing the medium with potassium hydroxide during blending did not improve the activity.

After blending, the preparation was squeezed through two layers of muslin. Most nuclei and larger pieces of cell debris were satisfactorily separated from

the homogenate by centrifuging for 5 min at 500 *g*. In early experiments the mitochondria were separated by centrifuging for 57 min at 1800 *g* or at forces greater than 10,000 *g* for a few minutes. When facilities for intermediate speeds became available centrifuging for 25 to 30 min at 5000 *g* was adopted as a standard procedure.

In some experiments the mitochondrial fraction was "washed" by resuspending in 50 ml of the blending medium and centrifuging for 40 min at 1800 *g*. A centrifuging time of 15 to 20 min at 5000 *g* was used for particles prepared at 5000 *g*. The endogenous oxygen uptake of mitochondria washed once was considerably lower than that of unwashed particles but was rarely reduced to zero. It was unnecessary to wash mitochondria even once to detect additional activity when some of the substrates were added.

For respiratory studies, the particles were resuspended in a small volume of a solution of the same composition as that in which the tissues had been blended. For ionic balance studies, the washed particles were resuspended directly in the medium in which they were to be studied. The preparation of the particles up to and including the stage of resuspension (for respiratory studies), and up to the stage of resuspension (for ionic studies) was carried out in the cold, in most experiments between 0 and 5°C.

(*b*) *Measurement of Respiratory Activity*

Oxygen uptake was measured by standard Warburg technique. The temperature of the thermostat was either 25 or 27°C. The temperature chosen was below that at which maximum activity could be obtained.

In all experiments all constituents of the medium were placed in the main compartment of the Warburg vessel. A total of 4 ml including 1 ml particle suspension, which was added last, was used in each vessel. After various trials to determine conditions for maximum activity it became standard practice to add cytochrome *c* (approx. $1{\cdot}6 \times 10^{-5}$M in vessel), phosphate buffer pH 7·0-7·4 (0·025M in vessel), and sucrose (15 per cent. in vessel) to all vessels, and substrate acids, neutralized with sodium or potassium hydroxide before adding, to some vessels. This mixture is conveniently referred to as "medium." Adenosine triphosphate and magnesium sulphate were added as required but not used in all experiments.

Oxygen uptake is expressed in several ways. For comparison within an experiment μl O_2/ml suspension/hr is a convenient expression. The best method of expression of results for comparison both within and between experiments is as μl O_2/mg nitrogen/hr. Nitrogen was estimated by the Nessler method after digestion of 0·5 ml mitochondrial suspension.

(*c*) *Ion Balance*

Investigation of the ion balance required that the mitochondrial particles be resuspended in the solutions in which they were to be investigated, aerated for various periods at 25°C, and then centrifuged and analysed for changes in ion concentration. The ions investigated were potassium, sodium, and chloride.

In some experiments the prepared particles were resuspended directly in the medium which was to be investigated. In others, the prepared particles were resuspended and "washed" by centrifuging before being resuspended in the medium to be investigated. The "washing" medium was varied according to requirements.

Particles were washed in six centrifuge tubes. The pellet in each tube was weighed and held at 0°C till required. The pellet in one tube was resuspended immediately in 5 or 3·7 ml of its own supernatant and centrifuged at once for 2 min at 25,000 *g* at 25°C. The pellet thus obtained was used to determine the concentration of ions in the particles initially. The pellets in the remaining tubes were each resuspended in 5 or 3·7 ml 15 per cent. sucrose (for loss experiments) or in medium containing substrate + KCl (for uptake experiments). At least one replicate was held in a Warburg vessel for measurement of oxygen uptake; in some experiments all were held in Warburg vessels to measure oxygen uptake. The remaining tubes were held, shaking for the required period at 25°C, and then centrifuged at once for 2 min at 25,000 *g* at 25°C. The suspension from the Warburg vessels was also centrifuged for 2 min at 25,000 *g* at 25°C.

Immediately after centrifuging, the supernatant was poured off and kept for analysis. The pellet was treated in the following way: (i) The tube was drained and the sides blotted with filter paper. (ii) the pellet was weighed and the particles resuspended in 6 or 4·5 ml water. (iii) A sample was removed for nitrogen estimation. (iv) The suspension was transferred to a test tube, weighed, heated in a boiling bath for 30 min to disorganize the particles, and weighed again. (v) This suspension was filtered, and the filtrate was then ready for chloride, potassium, and sodium estimations.

Chloride was determined by the electrometric titration method described by Best (1929), adapted for small samples, 0·5-1·0 ml, by use of an "Agla" micrometer syringe for the titration. The sample was acidified with nitric acid to suppress ionization of organic acids before titration.

Sodium and potassium were determined with a flame photometer as described by Domingo and Klyne (1949). The filtrate to be tested was diluted with 0·006M NaCl for K determinations and 0·006M KCl for Na determinations, and compared with standards of K in 0·006M NaCl and of Na in 0·006M KCl to overcome the interference of the two ions (Terner, Eggleston, and Krebs 1950). The accuracy of these determinations was ± 5 per cent.

Concentrations thus determined represent the concentration of ions in the mitochondrial pellet and, if the mitochondria accumulate the ions, represent an underestimate of the concentration within the particles themselves because an unknown amount of the supernatant solution will be packed between the particles. If the particles remain spherical, the amount of solution packed between them, with optimum packing, would be about 30 per cent. of the pellet volume and with less efficient packing may be 50 per cent. of the pellet volume. Added to this would be an unknown volume of liquid in the meniscus above the pellet. Bartley and Davies (1954) estimated the percentage of extra-

particulate water in their mitochondrial preparations as 66 per cent. Independent measurements (Hope, unpublished data) suggest that the extra-particulate water in our preparations was not more than 50 per cent.

IV. Results

(*a*) *Respiratory Activity*

(i) *Enzyme Activity of Different Fractions.*—Rapid oxygen uptake was obtained in cell-free extracts from beet and carrot tissue, with the substrates succinate, malate, aconitate, and pyruvate. Citrate was also tested but did not

Table 1

COMPARISON OF FRACTIONS FROM BEET TISSUE

Tissue (400 g) blended in 15% sucrose (400 ml), squeezed through muslin. Homogenate (590ml) centrifuged 5 min at 240 *g* giving 1st supernatant and 1st residue. Centrifuged 1st supernatant 1 hr at 1800*g* to give 2nd supernatant and 2nd residue. Portion of 2nd residue resuspended in 15% sucrose and centrifuged 40 min at 1800*g* to give 2nd residue, washed

Fraction	No Substrate	0·08M Sodium Succinate	0·08M Sodium Malate
	Oxygen Uptake (μl O_2/g fresh wt./hr)		
Whole disks	120-140	117	117
	Oxygen Uptake (μl O_2/ml fraction/hr)		
Homogenate	11	20	18
1st Supernatant	15	21	18
1st Residue	28	226	174
2nd Supernatant	8	11	10
2nd Residue	200	1680	1260
2nd Residue, washed	119	1180	716

increase the oxygen uptake. As has been shown with other plant tissues (Millerd *et al.* 1951) the activity of the enzymes which oxidize these substrates is associated with particles of approximately 1 μ diameter. This is clearly shown when various fractions are compared on the basis of activity per ml fraction (Table 1). No attempt was made to ensure complete recovery of the particles.

(ii) *Oxygen Uptake with Different Substrates.*—The oxygen uptake with different substrates was qualitatively the same with beet and carrot mito-

chondria. Results showing the oxygen uptake in the presence of different substrates are given in Tables 1 and 2 for beet and in Table 3 for carrot. The oxygen uptake with succinate was consistently high. In most experiments on beet tissue the oxygen uptake in the presence of malate was approximately 70

TABLE 2

OXYGEN UPTAKE BY BEET MITOCHONDRIA IN DIFFERENT SUBSTRATES

Centrifuging: 5 min at 500 *g*, 20-30 min at 5000 *g*

Experiment	Oxygen Uptake (μl O_2/ml/hr)			
	No Substrate	0·08M Sodium Succinate	0·08 Sodium Malate	0·08 Sodium Pyruvate
1	32·4	196	—	40
2	36	249	181	—
3	48	410	294	107
4	74	447	332	53
5	76	708	342	69
6	14·5	186	76*	—

* 0·04M Sodium malate.

per cent. of that in succinate while in carrot tissue it was only about 50 per cent. Increase of oxygen uptake with pyruvate was always very small but over the series of experiments the rate with pyruvate was greater than that with no

TABLE 3

OXYGEN UPTAKE BY CARROT MITOCHONDRIA IN DIFFERENT SUBSTRATES

Centrifuging: 5 min at 250 *g*, 40 min at 1600 *g*, 15 min (wash) at 1800 *g*. Substrate concentration: 0·04M

Added Factors	Oxygen Uptake (μl O_2/ml/hr)			
	No Substrate	Succinate	Malate	Aconitate
—	3	46	16	19
Phosphate, pH 6-7	9	47	26	18
Phosphate, pH 6-7 +Mg (0·001M) +ATP (0·0005M)	13	55	31	14

added substrate. Aconitate increased the oxygen uptake of carrot mitochondria; beet mitochondria were not tested with aconitate. Citrate did not increase but sometimes slightly depressed the oxygen uptake in both carrot and beet mitochondria.

(iii) *Oxygen Uptake under Different Conditions.*—(1) *Cytochrome c.* The presence of cytochrome *c* was not essential for an active oxygen uptake by mitochondria either with or without substrate. However, the oxygen uptake was enhanced by a small amount of cytochrome *c* as shown in Table 4.

TABLE 4

EFFECT OF CYTOCHROME *c* CONCENTRATION ON OXYGEN UPTAKE BY CARROT MITOCHONDRIA AT 27°C AND BY BEET MITOCHONDRIA AT 25°C

Blending medium: 15% sucrose. Expt. 1, 2, and 3: carrot mitochondria; centrifuging: 2 min and 40 min at 1150 *g*; substrate concentration 0·05M. Expt. 4: beet mitochrondria; centrifuging: 5 min at 500 *g*, 30 min at 5000 *g*; substrate concentration 0·08M

Experiment	Concentration of Cytochrome *c* in Vessel ($M \times 10^{-5}$)	Oxygen Uptake ($\mu l\ O_2$/ml/hr)	
		Sodium Succinate	Sodium Malate
1	0	23	—
	0·6	31	—
	1·3	30	—
	1·9	31	—
2	0	17	—
	1·0	32	—
	2·0	34	—
	2·9	33	—
3	0	21	—
	2·5	28	—
	3·8	24	—
4	0	406	144
	0·2	446	174
	0·4	494	206
	0·9	510	210
	1·8	522	220

(2) *Phosphate.* As with cytochrome *c*, the presence of phosphate was not essential for an active oxygen uptake by mitochondria but usually increased the uptake of oxygen with succinate as substrate (Table 5) and always with malate as substrate (Table 1). Experiments with increasing concentrations of phosphate showed that a low concentration (0·01M) was more than sufficient to give a maximum effect (Table 5).

(3) *Magnesium sulphate and adenosine triphosphate.* In several experiments these substances were added to the mitochondria in various combinations with phosphate, no added substrate, and pyruvate as substrate. Effects on oxygen uptake were small but variable, sometimes increasing and sometimes decreasing the rate. They were therefore not added as standard practice (but see also the next paragraph).

(4) *Catalytic Amounts of Malate.* Low concentrations of malate were tested in the presence of pyruvate as substrate (Table 6). Combination of substrate, malate in low concentration, and co-factors increased the oxygen

TABLE 5

OXYGEN UPTAKE OF BEET MITOCHONDRIA IN DIFFERENT CONCENTRATIONS OF PHOSPHATE WITH 0·06M SUCCINATE AS SUBSTRATE

Blending medium: water; centrifuging: 20 min at 1000 *g*

Preparation	Phosphate Concentration	Oxygen Uptake* (μl O_2/ml/hr)
1	0·024	65·8
	0·018	57·9
	0·012	57·4
	0·006	63·6
	0·001	54·4
	—	36·7
2	0·024	102·9
	0·018	86·6
	0·012	84·1
	0·006	97·7
	0·001	77·8
	—	68·2

*Mean of 2 readings.

uptake above the rates in no substrate and substrate alone. This may be a valuable combination for stabilizing these mitochondria and is being investigated further.

TABLE 6

OXYGEN UPTAKE (μLO_2/ML/HR) BY BEET MITOCHONDRIA WITH 0·017M PYRUVATE AS SUBSTRATE

Centrifuging: 5 min at 500 *g*, 20 min at 5000*g*; concentrations: malate 0·0001M, ATP 0·0005M, Mg 0·001M

No Substrate	Pyruvate	Malate	Pyruvate +Malate	ATP +Mg	Pyruvate +ATP +Mg	Malate +ATP +Mg	Pyruvate +Malate +ATP+Mg
73·8	60·8	65·4	71·4	97·2	97·2	93·6	111·0

(5) *pH.* An experiment in 0·025M phosphate showed no effect of pH over the range 6·8 to 7·6 on oxygen uptake in the absence of substrate and with pyruvate as substrate.

(*b*) *Ion Balance*

(i) *Sodium, Potassium, and Chloride in Mitochondria.*—Mitochondria which are extracted from cells have necessarily been exposed to a mixture containing electrolytes which are liberated from the cells into the blending medium. Since these electrolytes have come from both cytoplasm and vacuole, and have been diluted by the sucrose, this supernatant is different from that of the cytoplasm in which the mitochondria occur in intact cells. Since, as will be shown later, mitochondria adjust themselves relatively rapidly to their external solution, no measure of the ionic composition of mitochondria as they exist in intact cells has been obtained.

TABLE 7

CONCENTRATIONS OF SODIUM, POTASSIUM, AND CHLORIDE IN BEET MITOCHONDRIA IN mM/L AFTER EQUILIBRATION WITH THE EXTERNAL CONCENTRATIONS

The number of observations at each concentration is given in brackets. Means and standard errors have been calculated except when there are less than 4 observations

Na^+ External Concentration	Na^+ Pellet Concentration	K^+ External Concentration	K^+ Pellet Concentration	Cl^- External Concentration	Cl^- Pellet Concentration
0-6	22·8±2·6 (12)	0-6	11·0±0·8 (17)	0-2	4·9±0·6 (11)
7-13	39·6±3·9 (16)	7-13	20·6±1·9 (5)	5-12	11·7±0·7 (11)
		14-20	21·3±1·6 (5)	13-20	16·9±0·6 (18)
		22·5	28·7		
		23·4	23·8		
		22·8	26·1		
31·2	32·4	32·8	33·2		
33·5	35·6				
30·2	43·4				
		38·6	47·8	45-47	38·4, 39·0
		39·0	50·5		
		44·1	51·3		
		42·7	52·9		
		48·4	58·0		
		49·7	59·2		
79-85	88·1±2·6 (4)				
98·8	79·5				

(ii) *Equilibrium between Mitochondria and External Solution.*—The concentrations of ions released during blending varied with material from different sources, but analyses showed that the concentrations of sodium, potassium, and chloride ions in the mitochondria were greater than the respective concentrations in the supernatants. In a series of experiments the concentrations of sodium, potassium, and chloride were varied in the external medium after preparation and the mitochondria were allowed to adjust themselves to these concentrations before analysis. The concentrations in pellets and supernatants at the time of preparation and in pellets and external media

after adjustment are shown in Table 7. Since it was not always possible to control the external concentrations, the results for external concentrations have been grouped, the corresponding internal concentrations have been averaged, and the standard errors of the means calculated (except when there were less than 4 observations). The mean internal concentrations are plotted against the mid values in the external concentration ranges in Figures 1 and 2. At lower concentrations, the ratios of internal to external concentra-

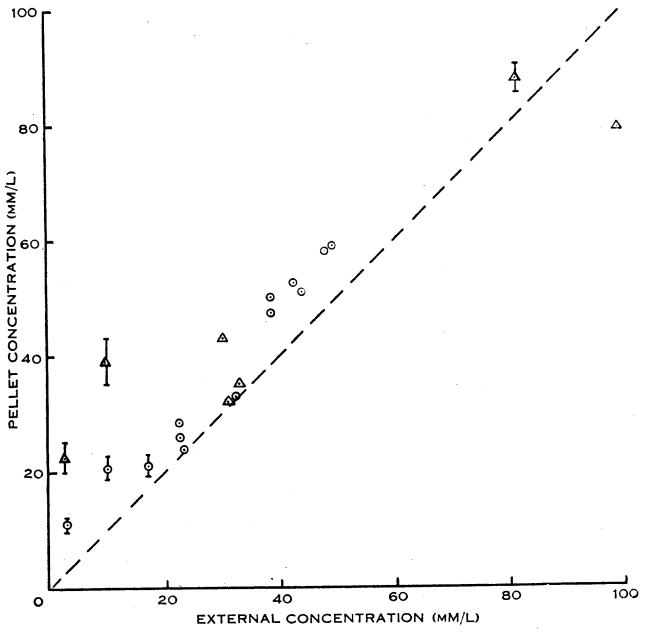

Fig. 1.—Concentrations of sodium and potassium in the pellet plotted against the concentrations in the external solutions. The dotted line shows equality of concentration. △, sodium; O, potassium.

tions are high for cations but approach unity at higher concentrations. The ratios of internal chloride to external concentrations are also high at low external concentrations but decrease and become less than unity for external concentrations greater than 18 mM/l. High ratios of internal to external concentrations for either mobile cations or anions would be expected if the adjustment were due to a Donnan equilibrium but, as will be discussed later, a high internal concentration of both mobile cations and anions cannot be explained as a simple Donnan effect. The internal concentration of sodium ions was higher than that of potassium and much higher than that of chloride.

(iii) *Time for Equilibration between Solution and Particles, and Diffusivity of Chloride in the Particle.*—Change in concentration of chloride when the mitochondria were transferred to higher or lower concentrations of chloride

was used to follow the form of the equilibration curve. Figure 3 shows equilibration curves for the transfer to a solution without chloride. Those for transfer to more concentrated solutions (are not plotted here but) have a similar equilibration period.

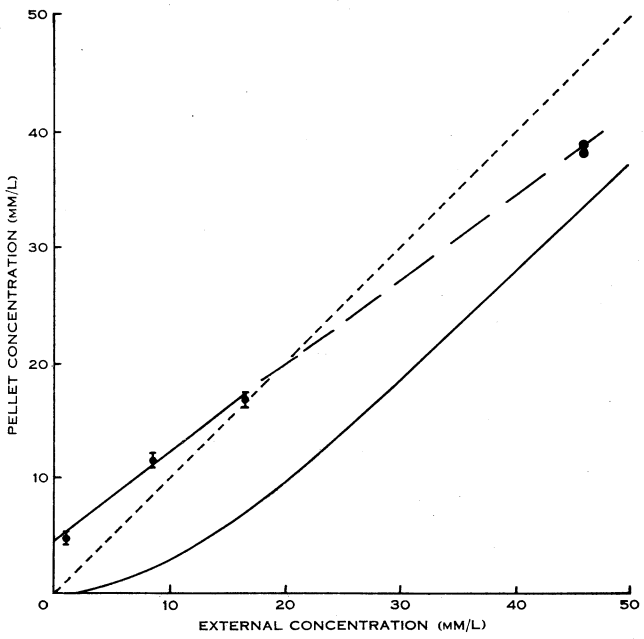

Fig. 2.—Concentration of chloride in the pellet plotted against the concentration in the external solution. The dotted line shows equality of concentration. The curve calculated from the Donnan equilibrium, assuming a concentration of immobile anions (A) of 30 mM/l, is also shown.

(iv) *Ionic Balance in the Mitochondria in Relation to Oxygen Uptake.*—The data given in Table 7 and Figures 1 and 2 show that the concentrations of cations and anions in the mitochondria exceed the concentrations in the external solution. A simple Donnan equilibrium will not explain the concentration of both positive and negative ions, but may be responsible for holding either the cations or the anions in the particles. One possible explanation would be that the proteins in the particles were behaving as anions and holding the cations; at ordinary pH values it seems unlikely that the proteins would be behaving as immobile cations holding the mobile anions.

The oxygen uptake of the pellet might be related to the internal concentration of ions in two ways; firstly, many intermediate compounds formed in metabolism are anionic and would therefore be expected to be balanced by cations, and secondly, one ion may be taken in by an accumulation mechanism dependent on the oxygen uptake. The difference between internal and external

concentrations after the steady state had been attained was measured simultaneously with the oxygen uptake of the particles. The oxygen uptake was examined in a Warburg vessel and the particles were then centrifuged (4 to 7 min) at

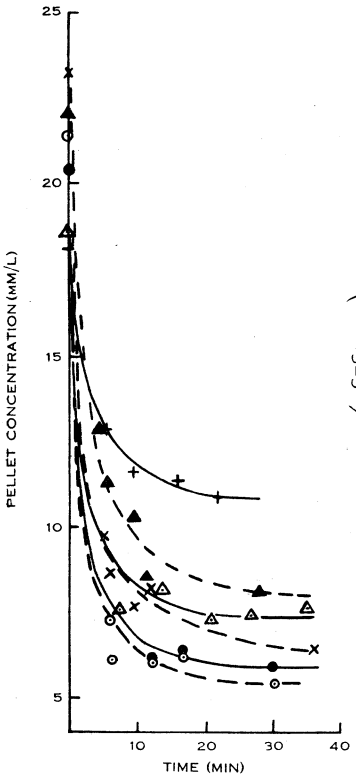

Fig. 3

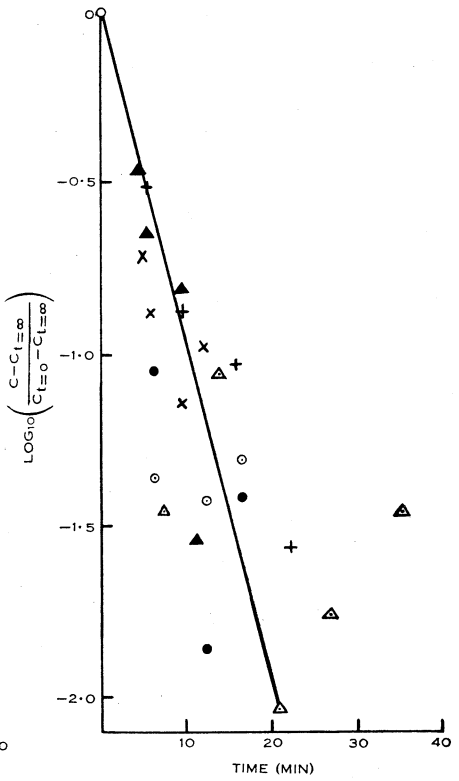

Fig. 4

Fig. 3.—The time-course of adjustment of pellet chloride concentration after change to a lower concentration in the external solution. Different symbols represent different experiments.

Fig. 4.—The data given in Figure 3 have been used to calculate $\log_{10}\left(\frac{c - c_{t=\infty}}{c_{t=0} - c_{t=\infty}}\right)$ (where c is concentration at time t, $c_{t=0}$ is the initial concentration, and $c_{t=\infty}$ is the final concentration), which has been plotted against time.

superspeed at about 25°C. The pellet thus obtained was analysed in the usual way for chloride, sodium, and potassium. The results are shown in Table 8 and Figure 5. Since the activity of different preparations can be compared only if the results are expressed as the amount of nitrogen which the pellet contains, the results are given as oxygen uptake/mg N and as concentration difference between pellet and supernatant/mg N in the pellet.

V. Discussion

(*a*) *Oxygen Uptake of Extracted Mitochondria*

The results confirm the observations of other workers that plant mitochondria can utilize acids of the Krebs organic acid cycle, bringing about increased oxygen uptake. The utilization of malic and succinic acids by the isolated mitochondria of carrot and beet suggests that the findings of Bennet-Clark and Bexon (1943) and of Turner and Hanley (1949) on tissue slices can be explained by the penetration of the substrates to the mitochondria of the whole tissue where they are utilized as substrates.

Table 8

COMPARISONS OF THE DIFFERENCE BETWEEN CONCENTRATIONS OF IONS IN THE MITOCHONDRIA AND SUPERNATANT WITH THE RATE OF OXYGEN UPTAKE

Experiment	Conditions	Oxygen Uptake (μl O_2/mg N/hr)	Differences between Internal and External Concentration					
			Na^+ (mM/l)	Na^+ (mM/mg N) ($\times 10^6$)	K^+ (mM/l)	K^+ (mM/mg N) ($\times 10^6$)	Cl^- (mM/l)	Cl^- (mM/mg N) ($\times 10^6$)
RJ 3	0·02M Malate, 0·010M KCl	80·4	55·5	43·3	11·5	9·0	6·4	5·0
	No substrate, 0·019M KCl	73	43·1	73·1	15·1	25·6	2·5	4·2
RJ 4	0·005M Malate, 0·014M KCl	129	29·2	22·3	10·4	7·9	3·7	2·8
	0·005M Malate, 0·014M KCl	108	30·1	22·9	10·7	9·9	1·3	1·2
	No substrate, 0·014M KCl	71·5	38·3	30·1	9·6	7·1	7·1	5·6
	No substrate, 0·014M KCl	39·5	30·8	27·5	8·3	7·4	1·9	1·7
HB 1	0·005M Malate, 0·014M KCl	66			6·2	3·6	4·2	2·4
		54·5			0·4	0·1	4·5	1·5
		75·4			3·3	1·3	1·6	0·6
	No substrate, 0·014M KCl	13·6			1·3	0·7	—	—
		25			1·5	0·6	1·2	0·5
		29·9			1·3	0·7	0·9	0·5
HB 3	0·005M Malate, 0·014M KCl	30·2	32·2	15·3	3·9	1·9	1·7	0·8
		51·3	11·3	7·6	6·6	4·4	6·4	4·3
		53	20·0	13·9	7·0	4·9	2·0	1·4
	No substrate, 0·014M KCl	11·6	5·3	3·8	4·0	2·9	0·2	0·2
		21·9	12·0	6·9	4·1	2·4	0·5	0·3
		10·2	12·0	7·2	6·1	3·6	1·5	0·9

Mitochondria from different plant sources after extraction appear to vary widely in their activity as measured by oxygen uptake when different substrate

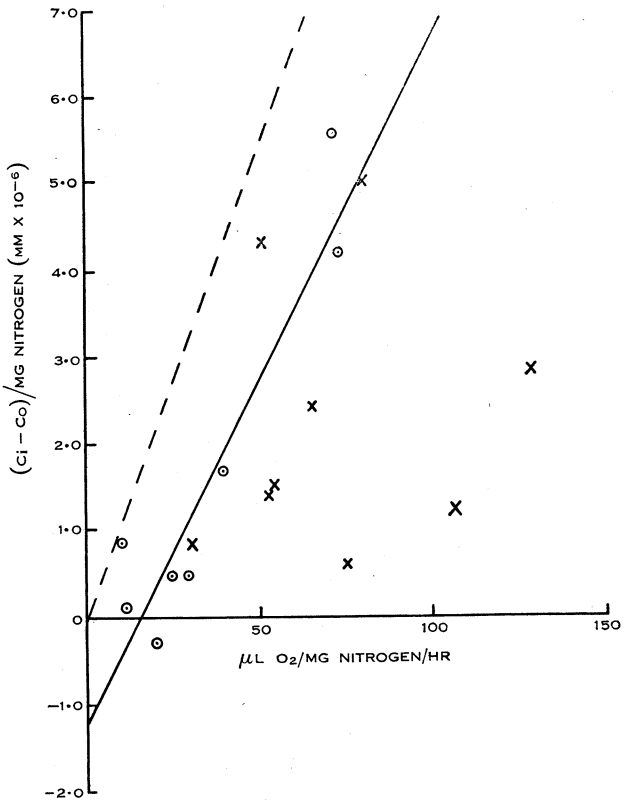

Fig. 5.—The chloride concentration difference between particles and external solution ($c_i - c_o$) plotted against the oxygen uptake. Both are expressed per mg N in the particles. ×, with substrate; ⊙, no substrate. The regression line calculated from the no substrate points is shown. The dotted line represents the expected relationship calculated from the hypothesis.

acids are added (Millerd 1952; Davies 1953; Laties 1953*b*). Increase in single step oxidation seems probable in our experiments in which the oxygen uptake with succinate as substrate is greater than that with any other substrate.

(*b*) *Physical Organization of Mitochondria*

The experiments on the adjustment to a new concentration of chloride establishes the fact that the mitochondrion offers a resistance to diffusion which makes the apparent diffusion constant very much less than that in water. If the

mitochondrion were treated as a homogeneous sphere the diffusivity of chloride in the body of the mitochondrion would be about 2×10^{-11} cm^2/sec which is much less than the diffusivity of chloride in water ($1{\cdot}67 \times 10^{-5}$ cm^2/sec at 20°C). Electron microscope studies, however, have shown that the plant mitochondrion, like the animal mitochondrion, is certainly not a homogeneous sphere (Palade 1952; Farrant, Robertson, and Wilkins 1953) but there is good evidence for an interfacial membrane of the order of 100-200 Å in thickness.

If it is assumed that the mitochondria are spheres approximately $1{\cdot}0\,\mu$ in diameter on the average, surrounded by a membrane about 200 Å in thickness (Farrant, Robertson, and Wilkins 1953) the apparent diffusion constant of the chloride in the membrane can be calculated from the equation

$$\log_{10}\frac{c - c_{t=\infty}}{c_{t=0} - c_{t=\infty}} = -\frac{3\,D\,t}{2{\cdot}303\,r\,l}, \quad \ldots \quad (1)$$

where $c_{t=0}$ is the internal concentration at zero time, c is the concentration at time t (in sec), $c_{t=\infty}$ is the equilibrium concentration of the particle, l is the thickness of the membrane (cm), and r is the radius of the particle (cm).

The best way of evaluating D is to plot the left-hand side of equation (**1**) against time (Fig. 4), measure the slope of the line of best fit, and solve the equation for D. From the results plotted, D is $1{\cdot}2 \times 10^{-13}$ cm^2/sec. As discussed earlier, however, the pellet concentration is an underestimate of the mitochondrial concentration because of the inter-particulate fluid. If the pellet contained 50 per cent. of inter-particulate fluid, the diffusion constant would be reduced to $0{\cdot}8 \times 10^{-13}$ cm^2/sec, and if it contained 70 per cent. of fluid, D would be reduced still further. Independent measurements suggest that the volume of inter-particulate fluid is about 50 per cent. It will therefore be adequate to take the diffusion constant of the mitochondrial membrane as being 1×10^{-13} cm^2/sec. This apparent diffusion constant can be used to apply Fick's law to calculate leakage rates from mitochondria when the concentration inside is higher than that outside. In calculating the diffusion constant it has been assumed that the morphological membrane (see Farrant, Robertson, and Wilkins 1954) is the main source of the resistance to diffusion of electrolytes in solution. If it were assumed that the resistance was uniformly distributed through the particle, the equation for diffusion in the homogeneous sphere could be used as an alternative and the principal conclusions would not be changed.

If there were no membrane present and diffusion were taking place into or out of a sphere of unstirred solution of similar dimensions to the mitochondrion, it can be shown that equilibration will be virtually complete in a millisecond. As seen from Figure 3, the equilibration time is of the order of minutes. Thus the layer of unstirred water which may surround the membrane of each mitochondrion has a negligible effect on the equilibration time compared with that of the membrane, and further, unless the diffusion constant inside the mitochondrion is very much less than in an aqueous solution, it is reasonable to assume that most of the resistance to diffusion is in the surface membrane. On

the assumption that most of the resistance to diffusion is located in this membrane, which is likely on the basis of the observed swelling properties of these mitochondria in dilute solutions and in water, the calculated diffusion constant of the chloride in this membrane is about 1×10^{-13} cm^2/sec. This approaches the order of the diffusion constant suggested by Danielli (1942) for electrolytes in a membrane of orientated lipoids stabilized by protein. A lipo-protein membrane has been suggested by Sjöstrand and Rhodin (1953) for the membrane of the mitochondria of mouse kidney epithelium. Further, impedance measurements on suspensions of beet mitochondria (Hope, unpublished data) suggest that the particles have a membrane of high impedance.

Although, as has been pointed out by Danielli (1942), Fick's law may not be applicable to very thin membranes where the resistance to diffusion is not necessarily proportional to the length of the diffusion path or thickness of the membrane, we have used the apparent diffusion constant for comparison with diffusion across other membranes of similar thickness. The permeability can be calculated from these values by dividing the diffusion constant by the thickness of the membrane.

The determination of the diffusion constant for chlorides in the mitochondrial membrane may point to several important conclusions, for example:

(i) It suggests that free ions inside the mitochondria as prepared are likely to diffuse from the mitochondria into the supernatant in about 10 min after extraction. We do not know how this diffusivity, determined after extraction, is related to diffusivity for the membranes in the unruptured cell. If, however, it is of the same order of magnitude, the mitochondrion probably adjusts itself fairly rapidly to its surrounding cytoplasmic medium. Soluble co-factors, if their diffusion constants were similar, would be lost from the particles on extraction, even though the integrity of the particles is largely preserved.

(ii) The low diffusion constant of chloride helps to explain the osmotic properties of the particles, which are apparently freely permeable to water. Presumably some of the molecules which have been used for maintaining the external concentration (e.g. sucrose) penetrate the membrane less rapidly than the chloride and thus, for a time, maintain the external osmotic pressure, preventing rapid water uptake by the particles.

The only experiments to establish similar permeability data for mitochondria seem to have been those of Cleland (1952) working with heart muscle sarcosomes. Using the reciprocal of the time (in minutes) for a 5 per cent. change in the density of the suspension which corresponded to a known tonicity difference, as a measure of the permeability, Cleland showed that the sarcosomes had the same permeability to potassium chloride, potassium succinate, potassium fumarate, potassium malate, and $H_2PO_4^-$ ions but the permeability to NaCl was about two-thirds of this and to sucrose about one-seventh. From data supplied by Cleland (personal communication), the half times of equilibration of these sarcosomes prepared without "Versene," and undergoing no oxidative phosphorylation, give a membrane diffusion constant of $0{\cdot}8 \times 10^{-13}$ cm^2/sec, assuming that the barrier to diffusion is mostly in the membrane. With "Versene," the calculated diffusion constant decreases to 1×10^{-14} cm^2/sec,

and with "Versene" and oxidative phosphorylation it decreases still further to the order of 2×10^{-15} cm^2/sec. So far no effects of "Versene" on the diffusion constant of chloride in plant particles have been found. The difference between sarcosomes and plant mitochondria means that the sarcosomes when undergoing phosphorylation (as they are in the intact cell) would probably require several hours to equilibrate to a change in their surroundings. We do not know whether there is a real difference between sarcosomes, which in the electron microscope show considerable internal structure, and plant mitochondria, which show little, or whether the difference is due to the extraction conditions leading to a greater disorganization of the plant mitochondria. This point requires further investigation, particularly as Chappell and Perry (1954) have shown recently that the swelling in hypotonic solutions of mitochondria from pigeon breast muscle can be reversed by the addition of ATP. These results together with those of Macfarlane and Spencer (1953) indicate the need for a full investigation to determine whether the control of swelling depends on phosphorylations influencing structure, or influencing ion uptake by the particles, or both.

(*c*) *Mechanism of Maintaining Internal Concentrations of Ions*

When both positive and negative mobile ions of a salt are held in higher concentration in one phase than another, no simple Donnan equilibrium can explain the distribution, as was shown by Briggs and Petrie (1928) in a discussion of ion accumulation by the whole tissue. Bartley and Davies (1954) have shown that, as in our results, mobile anions and cations in the mitochondria of sheep kidney, exceed the concentration of the external solution. There are three possible explanations of such a situation: (i) the mobile ion of one charge is bound and removed from the aqueous phase inside the cell or particle while the mobile ion of the other charge is held by the immobile ions of opposite charge; (ii) there are two phases inside the cell or particle, one containing immobile anions balanced by the mobile cations, and the other containing immobile cations balanced by the mobile anions but the two phases must be quite distinct; (iii) one ion could be held by a simple Donnan equilibrium while the other ion is maintained by some accumulating mechanism against the concentration gradient.

There is insufficient evidence to choose definitely between these three explanations. Stanbury and Mudge (1953) found that potassium could be retained by liver mitochondria against a concentration gradient and that it was only slowly exchanged under their experimental conditions. However, as Bartley and Davies (1954) have shown, the major portions of the ions held under conditions of active metabolism by the particles are easily exchangeable and we are dealing here with actively metabolizing particles. If compounds which would remove either the cations or the anions from solution exist, they must be destroyed upon the disintegration of the particle. While the second explanation is not excluded, there is at present no evidence in its favour. Plant mitochondria do not seem to be characterized by the transverse internal partions seen in animal mitochondria (Sjöstrand and Rhodin 1953).

The electrophoretic mobility (McLean, unpublished data) of the plant mitochondria prepared in this Laboratory suggests that the particle has a large surface excess of immobile negative charges. It seems that the third possibility, i.e. that the cation is held by the Donnan effect while the anion is held by an accumulatory mechanism working against the leakage merits further investigation. The concentration of immobile anions due to proteins, phospho-lipids, and other substances such as intermediates of metabolism would balance the excess concentration of sodium and potassium which has been observed inside. This concentration of cations tends to be higher with higher respiration rates (Table 8). It is not clear at present why the concentration of Na^+ should be so much higher than that of K^+. Similar higher concentrations of cations were observed by Bartley and Davies (1954) and were shown to depend on the metabolic activity of the particles.

The full analysis of the relation between internal and external concentrations of cations is impossible without knowledge of the concentration of immobile anions and of the competing power of sodium and potassium for these anions. An estimate of the probable concentration of the immobile anion (A) can be obtained from the apparent intercepts of the internal sodium and potassium concentrations at zero external concentration, allowing for the cations paired with the chloride at zero. This value is approximately 0·03M. If the Donnan equilibrium were the only factor determining the concentration of the ions, the expected internal chloride concentration, $[Cl_i]$, neglecting other mobile anions, can be calculated from the equation

$$[Cl_i] = \tfrac{1}{2}[(A^2 + 4c_0^2)^{\frac{1}{2}} - A], \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

where c_0 is the external concentration of chloride. The expected internal concentrations of chloride are shown in Figure 2, where it can be seen that the internal concentrations of chloride at lower external concentrations are considerably in excess of those expected from a simple Donnan equilibrium. At higher concentrations the internal chloride could be consistent with the Donnan effect being mainly responsible.

Some other mechanism must therefore be suggested to explain the accumulation of mobile anions. The anion concentration difference is not as great as that of the cation. From the diffusivity of Cl^- already determined, the leakage rate if the particle were free in solution in the mitochondria can be calculated from the equation

$$-\frac{dc}{dt} = \frac{3D}{rl}(c_i - c_0), \quad \ldots \quad \ldots \quad \ldots \quad (3)$$

where $(c_i - c_0)$ is the concentration difference between inside and outside (from our experiments about 3 mM/l $= 3 \times 10^{-6}$ g-mol/c.c.), D is the diffusivity of Cl^- in the membrane (1×10^{-13} cm^2/sec), r is the radius of the particle ($0{\cdot}5 \times 10^{-4}$ cm), and l is the thickness of the membrane (2×10^{-6} cm). The rate of leakage from the mitochondria would be then 9×10^{-9} g-mol/c.c./sec. Since each pellet of mitochondrial suspension weighs about 0·14 g, i.e. is approximately 0·14 ml, the leakage rate/pellet would be $4{\cdot}5 \times 10^{-6}$ g-mol/pellet/hr. If now this concentration difference is maintained over some time, in the steady

state, this leakage rate must be balanced by an equal accumulation rate, i.e. $4{\cdot}5 \times 10^{-6}$ g-mol Cl^-/pellet/hr.

As mentioned in the introduction, the hypothesis that the accumulation mechanism depends on the transfer of an anion into the cell when an electron passes through the electron carrier system has been shown to be consistent with the quantitative data for accumulation rates and respiration rates in whole tissue (Robertson and Wilkins 1948). It seemed worth investigating whether the accumulation rate, shown above to be necessary for the observed concentration difference between mitochondria and surrounding solution, would have the right quantitative relation to the rate of oxygen uptake. The hypothesis that the accumulation mechanism depends on the transfer of an anion by an electron carrier requires that the maximum accumulation rate should approximate to four times the oxygen uptake in g-mol (since 4 electrons are required in the reduction of one oxygen molecule to water). Thus an accumulation rate of $4{\cdot}5 \times 10^{-6}$ g-mol/pellet/hr would require an oxygen uptake of $1{\cdot}1 \times 10^{-6}$ g-mol O_2/pellet/hr or $27{\cdot}5\,\mu$l O_2/pellet/hr. Since the average pellet weighs about 0·14 g and contains about 1·4 mg nitrogen, the expected relationship between the concentration difference $(c_i - c_0)$/mg N in the pellet and the oxygen uptake/mg N/hr can be calculated and is shown in Figure 5. This figure also shows the difference between internal and external chloride concentrations of the experiments in Table 8. When no substrate is added, the correlation of the difference between internal and external concentration with the oxygen uptake is high ($r = 0{\cdot}913$, $P < 0{\cdot}01$); the regression line is shown in Figure 5. When substrate is added the correlation with oxygen uptake is low ($r = 0{\cdot}15$, $P > 0{\cdot}5$), and therefore not significant. Thus the difference between internal and external concentrations is of the order of magnitude which would be expected if the accumulation of chloride by the hypothetical mechanism were off-setting the leakage, and the correlation with oxygen uptake is high. Theoretically it would be better to examine the relation of the difference between internal chloride concentration and that calculated from the Donnan equilibrium, to the oxygen uptake; uncertainty about the correction for interparticulate fluid and about the concentration of immobile anions (A) make this impossible with the present data. The extrapolate of the experimental curve (Fig. 5) to less than zero concentration difference at zero oxygen uptake is to be expected from the Donnan equilibrium.

Two reasons can be suggested for the substrate experiments tending to show lower concentration differences for a given oxygen uptake: (i) the substrate supplied externally may introduce a number of single-step oxidations which are not as effective as the endogenous hydrogen transport in the accumulatory mechanism, and (ii) with substrate, the accumulation judged by chloride concentration may be less than the total accumulation because substrate anions may be competing with the chloride. Cleland's quoted results show that there is little difference between the rates of equilibration of chlorides and organic acid anions in sarcosomes, and Bartley and Davies (1954) have shown that the organic acid anions are accumulated by kidney mitochondria.

Thus mitochondria can be interpreted as having a high concentration of immobile anions responsible for the higher concentrations of mobile cations in the particle than in the external solution. Simultaneously an anion accumulation mechanism may operate across a membrane of high resistance. The concentration of anions thus maintained is consistent with that expected if the leakage through the membrane were balanced by an uptake of anions equivalent to the electron transport. The transport of anions would depend upon the cytochrome system, which, according to the arguments of Palade (1952), is probably located in the membrane. In such a position the cytochrome system may operate to transfer the anions across the membrane provided that the combination was so complete that an un-ionized complex was formed during the period of transport. This work indicates not only that the quantitative relations would be consistent with this hypothesis but that the anion accumulated is chloride, which is not recognized as entering directly into any of the known biochemical reactions of the mitochondria. The accumulation of the organic acid anions demonstrated by Bartley and Davies (1954) might be interpreted as being due to these anions being worked into the metabolic pool in which they participate, though accumulation may take place by the same mechanism as suggested here for chloride.

(*d*) *Mitochondria and Salt Accumulation in Plants*

The probable role of mitochondria in salt accumulation was discussed by Robertson (1951), who pointed out that the mitochondrion, if capable of accumulating ions, would be a suitable body to transfer ions across the cytoplasm and into lipoid regions because not only do mitochondria have a lipoprotein structure but they are also moved effectively in the protoplasmic streaming. The results reported in this paper support this hypothesis. The mitochondrion, actively oxidizing substrate in one part of the cell, may increase its concentration of mobile ions and then, in cytoplasmic streaming, be moved to another part of the cell, where if its oxidative activity decreases, the ion concentration in the particle will also decrease. If the movement of the particle has been through a region of low permeability, the ions liberated from the particle will diffuse to the cell surface again only slowly. Such a low permeability could be due either to a high concentration of immobile ions in the cytoplasm, which would lower the diffusivity for Donnan reasons, or to a region of high lipoid content. Microscopic examination of the cytoplasmic streaming shows that the mitochondria are in frequent contact with the inner layers of the cytoplasm.

Some recent work (Epstein and Hagen 1952; Epstein 1953; Scott Russell, Martin, and Bishop 1953) has concentrated on the hypothesis that carriers with some degree of specificity are concerned in combinations with the ions in the cytoplasm and their transport into the cell. If such carriers exist, their location in specific places in the cell is important and in this connection the present work on sodium and potassium distribution in mitochondria is interesting.

(*e*) *Mitochondria and Electrolytes in Other Cells*

The accumulatory capacity of mitochondria is probably important to tissues other than plant cells. The suggestions for the secretory activity of mitochondria in animal cells, discussed by Zollinger (1950), have been summarized recently by Lindberg and Ernster (1954). The present paper emphasizes the possibility that a definite ion transport mechanism exists in the mitochondria, related to the respiration and made possible by the membrane by which most mitochondria seem to be surrounded. Recent discussions of the gastric secretion of hydrochloric acid (Conway 1952; Davenport 1952) show that the quantitative relations are consistent with the hydrogen ions being derived from the hydrogens of respiration separated from the electrons which pass over the oxidase system. If the mitochondria of the secreting oxyntic cells have a membrane which is predominantly impermeable to cations and allows the electrons to pass in exchange for anions (e.g. Cl^-), the same secretory principle, dependent on mitochondrial structure and function, underlies both hydrochloric acid secretion and salt accumulation in plants.

VI. Acknowledgments

The work described in this paper was carried out as part of the joint research programme of the Division of Food Preservation and Transport, C.S.I.R.O., and of the Botany School, University of Sydney. The authors wish to express their thanks to Miss J. Houguet for technical assistance, to Dr. S. I. Honda for assistance with some of the experiments, to Professor J. S. Turner, Botany School, University of Melbourne, Dr. R. K. Morton, Biochemistry School, University of Melbourne, Dr. K. W. Cleland, Department of Anatomy, University of Sydney, Mr. E. W. Hicks, and Dr. F. V. Mercer for their helpful criticism of the manuscript, and to Dr. J. R. Vickery, Chief of the Division of Food Preservation and Transport, and Acting-Professor J. McLuckie, Botany School, University of Sydney, in whose laboratories the work was carried out.

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