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The Effects of Manganese on the Solute Content of Rat-liver Mitochondria

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Manganese is known to affect the metabolic activities of isolated mitochondria (see e.g. Lindberg & Ernster, 1954) and also to prevent the loss of ultraviolet-light-absorbing substances from the mitochondria (Siekevitz & Potter, 1955). In view of these observations it was thought desirable to study the effect of Mn^{2+} ions on isolated mitochondria under a variety of conditions.

MATERIALS AND METHODS

Special chemicals

Sodium 1-azo-2-hydroxy-3-(2:4-dimethylcarboxanilido)-naphthalene-1′-(2-hydroxybenzene-5-sulphonate), used in the estimation of Mg^{2+} ions by the method of Mann & Yoe (1956), was purchased from LaMotte Chemical Products Co., Baltimore, Maryland, U.S.A. Ethylenediaminetetra-acetic acid (EDTA), formaldoxime hydrochloride and 8-hydroxyquinoline (AnalaR) were obtained from British Drug Houses Ltd.

Mitochondrial fractionation

This followed the procedure of Werkheiser & Bartley (1957). The mitochondrial pellet was suspended in sufficient 0·25 M-sucrose to give about 350 mg. wet wt. of mitochondria/ml. Usually 1 ml. of this suspension was added to 4 ml. of 0·25 M-sucrose or 4 ml. of 0·125 M-KCl, either of which contained $MnCl_2$, usually m-molar in the final suspension. After incubation, a portion (2 ml.) of the mixture was centrifuged, usually for 10 min. at 24 000 *g*. The supernatant fluid was decanted into 15 ml. conical centrifuge tubes containing 1 ml. of 30% trichloroacetic acid. After clarification by centrifuging the supernatant was set aside for analysis. The treatment of the mitochondrial pellet for the determination of the acid-soluble solutes and the acid-insoluble dry matter was described by Werkheiser & Bartley (1957). All analytical data refer to the samples (2 ml.) which were taken for centrifuging.

Analytical methods

Potassium was measured with a lithium internal standard flame photometer (Amoore, Parsons & Werkheiser, 1958). Manganese was measured by the use of formaldoxime (Bartley, Notton & Werkheiser, 1957). Calcium was estimated flame-photometrically with the apparatus of Exley (to be published) and chloride by the method of Sanderson (1952) with the slight modifications introduced by Amoore & Bartley (1958). The ultraviolet absorption at 260 mμ of the $HClO_4$ extracts of the supernatant medium was measured in a quartz cell with a 1 cm. light path in a Beckman spectrophotometer. From these measurements the absorption due to these substances in the original medium was calculated. The changes in absorption at this wavelength were assumed to be due to changes in the nucleotide content of the solutions. The estimation of the water content, and of the dry matter non-extractable with trichloroacetic acid, of the mitochondrial pellet was according to Werkheiser & Bartley (1957). Total phosphate was estimated by the method of Berenblum & Chain (1938), as modified by Bartley (1953), on a sample of the extract after wet-ashing according to Hanes & Isherwood (1949).

Measurements of pH were made with the Beckman probe assembly, type 14400, and a Beckman pH meter, model G. Measurements were made on the suspensions remaining after the sample (2 ml.) had been abstracted for centrifuging. The temperature of measurement was that at which the mitochondria had been incubated.

Magnesium was estimated either by the modification of the colorimetric method of Mann & Yoe (1956) described by Amoore & Bartley (1958) or by the flame-photometric method of Exley (to be published). Mn^{2+} ion interferes in

the colorimetric method by decolorizing the dye. The interference could be overcome either by causing the Mn^{2+} ions to form a complex with 8-hydroxyquinoline and extracting it into $CHCl_3$ (a slight modification of the procedure of Mann & Yoe, 1956) or by causing it selectively to form a complex with EDTA.

Removal of Mn^{2+} *ions by extraction.* To the extract in trichloroacetic acid (0·1 ml. of the mitochondrial extract or 0·5 ml. of the supernatant) in a 10 ml. graduated tube was added 0·5 ml. of conc. HNO_3. The mixture was slowly evaporated to dryness (taking about 2 hr.) and the residue was taken up in 2 ml. of 5 mM-HCl. To this was added 1 ml. of borate buffer (0·08 M-sodium borate, pH 9·2) and 0·1 ml. of 5% 8-hydroxyquinoline in $CHCl_3$. Chloroform (5 ml.) was immediately added and the tube was stoppered and shaken vigorously for about 5 sec. The lower $CHCl_3$ layer was carefully withdrawn by means of a Pasteur pipette and discarded. The extraction with $CHCl_3$ was repeated a further four times, then the magnesium dye was added and the procedure continued according to the modification of the method of Mann & Yoe (1956) without further addition of borate buffer.

Removal of Mn^{2+} *ions by ethylenediaminetetra-acetic acid.* To the samples of tissue extract was added sufficient EDTA to chelate all the manganese in the sample. It is immaterial if a slight excess of EDTA is added. Instead of the normal amount of calcium (0·2 μmole) 0·3 μmole was added to remove any excess of EDTA and still leave sufficient free Ca^{2+} ions to produce the enhancement of colour described by Mann & Yoe (1956; see also Amoore & Bartley, 1958). The sample was adjusted to 2 ml. and the subsequent procedure was that used for Mg^{2+} ions in the absence of Mn^{2+} ions.

Use of Titan yellow for the estimation of Mg^{2+} *ions in the presence of sucrose or* Mn^{2+} *ions.* In some earlier experiments a Titan yellow method (Garner, 1946; see also Siekevitz & Potter, 1955) for the estimation of Mg^{2+} ions was found to give erroneous results in the presence of sucrose or Mn^{2+} ions. Recoveries as low as about 61% occurred when the sucrose content of the test sample was 112 μmoles and only 90% recovery was obtained in the presence of 7·5 μmoles of sucrose. The phenomenon was not further studied because the more convenient and sensitive method of Mann & Yoe (1956) became available. Siekevitz & Potter (1955) do not mention any interference by sucrose; it is possible that there is no interference when polyvinyl alcohol is the stabilizer instead of gum ghatti. Whereas the interference by sucrose is comparatively small, the interference due to Mn^{2+} ions is gross and unpredictable. Mn^{2+} ion gives an apparent reaction for Mg^{2+} ion with Titan yellow. This is due to the formation of a dark precipitate, probably MnO_2, on the addition of the concentrated alkali. In a mixture of Mn^{2+} and Mg^{2+} ions the absorptions of the two ions in the Titan yellow test are not additive and an unpredictable optical density may be obtained. If the amount of Mn^{2+} ions added was large in comparison with the Mg^{2+} ions added the Mg^{2+} ions were grossly overestimated, but if the amounts of the two ions were more nearly equal, a large overestimation did not occur but, in some cases, an underestimation. It is not known if the use of Titan yellow, according to the procedure of Orange & Rhein (1951), as used by Siekevitz & Potter (1955), results in the same errors.

Duplicate estimations of K^+, Cl^-, Mn^{2+} and Mg^{2+} ions, phosphate, dry matter and water content agreed to better than 2% under the experimental conditions. According to Dr D. Exley (personal communication) duplicate estimations of Ca^{2+} and Mg^{2+} ions by his flame-photometric technique differ by not more than ±3% at the levels found in our experimental material.

RESULTS

Effect of manganese on the colour of the mitochondrial pellet. Mitochondria freshly prepared and sedimented at 0° yield a pale-buff pellet. On warming to room temperature the colour of the pellet changes within 1–2 min. to dark brown. As the brown colour develops, the mitochondrial pellet becomes increasingly translucent. On the other hand, when the mitochondria were obtained as a sediment from suspension media containing mM-Mn^{2+} ions, the pellet only very slowly turned brown on warming, taking up to 3 hr. when the suspension medium was 0·25 M-sucrose with Mn^{2+} ions and a somewhat shorter time when the medium contained 0·1 M-KCl and 0·05 M-sucrose. Thus the Mn^{2+} ions seem to preserve the mitochondria from disintegration.

Effect of manganese on the release of ultraviolet-light-absorbing material and on change of mitochondrial dry weight during incubation in 0·25 M-*sucrose solution.* The experiment shown in Table 1

Table 1. *Effect of manganese on the leakage of nucleotides from mitochondria*

Rat-liver mitochondria were prepared and suspended in 0·25 M-sucrose and were incubated at 21° alone or with the addition of $MnCl_2$ (final concn. mM). Samples of the suspension were centrifuged at the times given for subsequent analysis of the mitochondrial pellet and the medium. All analytical data refer to the samples (2 ml.) taken for centrifuging. The optical density was measured on the deproteinized solution and values were then expressed in terms of the volume of the original undiluted medium.

Incubation time (min.)	Concn. of Mn^{2+} ions in medium (mM)	Dry wt. of pellet (%)	Calc. optical density of medium at 260 mμ after precipitation of protein
0	0	33	0·277
5	0	32·3	0·510
10	0	27·9	0·895
15	0	27·6	1·224
20	0	26·9	1·287
40	0	26·6	1·305
60	0	26·6	1·326
0	1	36·6	0·194
5	1	38·0	0·364
10	1	38·0	0·382
15	1	34·3	0·370
20	1	33·1	0·390
40	1	30·4	0·478
60	1	31·7	1·104

confirms the results of Siekevitz & Potter (1955) that in the presence of mM-Mn^{2+} ions the leakage of substances absorbing at 260 mμ was greatly reduced. Table 1 also shows that the addition of the Mn^{2+} ions caused an immediate decrease in the water content of the mitochondrial pellet. Throughout the incubation, the % dry wt. of the mitochondrial pellet in the solution containing Mn^{2+} ions (cf. Tapley, 1956) was higher. In a similar experiment shown in Table 2 it was further shown that mM-Mn^{2+} ions also retarded the loss of material (presumably largely protein) precipitable with trichloroacetic acid from the mitochondria.

Effect of manganese on the release of ultraviolet-light-absorbing material and on the percentage of mitochondrial dry weight during incubation in solutions containing 0·05 M-sucrose and 0·1 M-potassium chloride. *In vivo*, the mitochondria are exposed to the high intracellular concentration of potassium, and in most metabolic experiments *in vitro* the mitochondria are suspended in media containing potassium. In these ionic media the

Table 2. *Manganese uptake of mitochondria and loss of nucleotide and protein*

The mitochondria were prepared and incubated as in Table 1. Optical density is expressed as in Table 1.

Incubation time (min.)	Mn^{2+} ions added to medium (μmoles)	Dry wt. of pellet (%)	Mn^{2+} ions in medium after incubation (μmole)	Calc. optical density of medium at 260 mμ after precipitation of protein	Protein in medium after incubation (mg.)
0	0	28·61	0	0·313	1·7
5	0	27·06	0	0·479	2·5
10	0	27·29	0	0·411	3·3
15	0	28·02	0	0·501	4·4
20	0	28·19	0	0·540	4·8
40	0	27·7	0	0·617	7·5
60	0	26·8	0	0·699	8·8
0	2	32·1	0·75	0·259	1·2
5	2	30·38	0·75	0·304	1·6
10	2	29·69	0·76	0·338	1·7
15	2	29·51	0·795	0·348	2·1
20	2	29·54	0·776	0·369	2·5
40	2	29·41	0·82	0·450	3·4
60	2	29·34	0·785	0·487	4·0

Table 3. *Effect of manganese on dry weight and nucleotide content of rat-liver mitochondria suspended in 0·1 M-potassium chloride and 0·05 M-sucrose*

The mitochondria were prepared in 0·25 M-sucrose, resuspended in the KCl–sucrose medium and incubated at the temperatures given below. Optical density is expressed as in Table 1. Analytical data refer to the samples (2 ml.) taken for centrifuging.

Temp.	Incubation time (min.)	Mn^{2+} ions added to medium (μmoles)	Dry wt. of pellet (%)	Calc. optical density of medium at 260 mμ
0°	0	0	29·46	0·738
	10	0	29·92	0·960
	20	0	29·8	1·044
	40	0	28·8	1·2
	60	0	28·61	1·416
20·7	5	0	28·18	1·374
	10	0	27·98	1·794
	20	0	26·82	2·658
	30	0	24·08	3·234
0	0	2	31·49	1·044
	10	2	31·21	1·128
	20	2	30·8	1·158
	40	2	30·47	1·284
	60	2	30·1	1·662
20·7	5	2	30·2	1·554
	10	2	30·16	2·256
	20	2	28·23	3·468
	30	2	23·55	3·918

Table 4. *Effect of manganese concentration on its uptake by rat-liver mitochondria suspended in 0·25 M-sucrose solution*

The mitochondria were prepared and maintained in 0·25 M-sucrose at 0°. The water content of the mitochondria before addition of Mn^{2+} ions was 2·83. Manganese chloride was added to give the amounts shown below and the mixture was kept at 0° for 15 min. before centrifuging. Analytical data refer to the sample (2 ml.) taken for centrifuging.

Mn^{2+} ions added to medium (μmoles)	Content of Mn^{2+} ion of mitochondrial pellet (μmoles)	Content of Mn^{2+} ion of dry mitochondrial pellet (m-moles/kg.)	Concn. of Mn^{2+} ion in water of pellet (m-molal)	Concn. of Mn^{2+} ion in medium (m-molal)	Concn. of Mn^{2+} ion in pellet/concn. of Mn^{2+} ions in medium	Water content of mitochondria (l./kg. dry wt.)
2	1·43	25·6	9·66	0·37	26·1	2·66
4	2·44	43·0	18·6	0·96	19·4	2·31
10	3·58	60·0	27·4	3·83	7·2	2·20

behaviour of the mitochondria is different from that in sucrose alone; for example, their adenosine triphosphatase activity stimulated by dinitrophenol is greatly enhanced (Lardy & Wellman, 1953). Accordingly, the effect of Mn^{2+} ions was tested in a medium containing 0·05 M-sucrose and 0·1 M-KCl. As shown in Table 3, Mn^{2+} ions no longer prevented the loss from the mitochondria of substances absorbing at 260 mμ (E_{260} substances). The rate of loss of these substances was greatly accelerated by raising the temperature of the suspending medium to 20°. At 0°, Mn^{2+} ions still maintained the water content of the mitochondrial pellet at a lower level than the control pellets without Mn^{2+} ions; this also occurred on incubation at 20°, but if incubation was prolonged beyond 20 min. at this temperature, both sets of mitochondria swelled to the same water content.

Uptake of manganese by mitochondria suspended in 0·25 M-sucrose solution. Siekevitz & Potter (1955) gave no measurements of the distributions of Mn^{2+} ions between the particles and the medium. The data of Maynard & Cotzias (1955) suggest that there would be little uptake of Mn^{2+} ions by the mitochondria *in vitro*. However, our experiments showed that more than one-half (1·25 μmoles, see Table 2, zero time with Mn^{2+} ions) of the Mn^{2+} ions was taken up by the mitochondria in the 5 min. at 0° that elapsed between adding the Mn^{2+} ions and warming the suspension to 20°. Incubation at this temperature did not result in a further uptake of Mn^{2+} ions.

Siekevitz & Potter (1955) showed that mitochondria suspended in media containing Mn^{2+} ions at a higher concentration than 2 mM lost their ability to carry out oxidative phosphorylation. In Table 4 is shown the uptake of Mn^{2+} ions by mitochondria from solutions containing 1, 2 and 5 mM concentrations of this ion. The amount of Mn^{2+} ions taken up increased with the external concentration but not proportionally to it. The maximum concentration gradient was obtained with the lowest concentration of Mn^{2+} ions used. These results contrast with those of Maynard & Cotzias (1955), who found only a 10% uptake of Mn^{2+} ions at the very low concentration of Mn^{2+} ions (0·03 mM) that they used. However, it will be shown that prolonged incubation at room temperature in the absence of substrate leads to a loss of the Mn^{2+} ions that was previously taken up (see Fig. 1), and this may explain their low uptake.

As shown already, the addition of Mn^{2+} ions to the mitochondria resulted in a loss in the water content of the pellet. More water was lost when the content of Mn^{2+} ions was increased, but there was no simple relationship between loss and the external concentration of Mn^{2+} ions (Table 4).

Uptake of manganese by mitochondria suspended in 0·1 M-potassium chloride and 0·05 M-sucrose. As Mn^{2+} ions had no effect on the loss of E_{260} substances when the mitochondria were suspended in the KCl-containing medium, it might have been expected that uptake of Mn^{2+} ions from this medium was inhibited. However, as shown in Table 5, the uptake of Mn^{2+} ions from this medium was much greater than from sucrose alone, reaching 35–40 m-moles of Mn^{2+} ions/kg. of dry mitochondrial pellet in between 10 and 20 min., compared with 25 m-moles of Mn^{2+} ions/kg. of dry mitochondrial pellet in mitochondria incubated for the same time in 0·25 M-sucrose. After 1 hr. at 0° the content of Mn^{2+} ions of the pellet had risen to 57 m-moles/kg. dry wt.

At 21° the rate and amount of uptake of Mn^{2+} ions were still further accelerated. Within 5 min., more than 60% of the added Mn^{2+} ions were taken up and after 30 min. the content of Mn^{2+} ions reached 81 m-moles/kg. dry wt. of mitochondria. If this amount of Mn^{2+} ions is considered to be dissolved in the mitochondrial water the concentration is 23·3 mM, a ratio (Mn^{2+} ions of mitochondria: Mn^{2+} ions of medium) of 185:1. In the experiments so far described the mitochondria were added to a mixture of KCl and $MnCl_2$ solutions and thus the two cations might have competed for penetration of the mitochondria. The uptake of Mn^{2+} ions by mitochondria that had been equilibrated with 0·1 M-KCl before the addition of Mn^{2+} ions was

Table 5. *Uptake of manganese by mitochondria suspended in* 0·1 M-*potassium chloride and* 0·05 M-*sucrose*

The mitochondria were prepared and incubated as in Table 3.

Incubation time (min.)	Temp.	Concn. of Mn^{2+} ions in water of		Content of Mn^{2+} ions of dry mitochondrial pellet (m-moles/kg.)	Concn. of Mn^{2+} ions in pellet/concn. of Mn^{2+} ions in medium
		Pellet (m-molal)	Supernatant (m-molal)		
0	0°	10·9	0·63	24·8	17·3
10	0	15·0	0·51	34·6	29·5
20	0	17·5	0·42	41·2	41·4
40	0	22·2	0·29	53·1	76·0
60	0	23·4	0·24	56·9	95·7
5	21	18·3	0·37	44·0	49·6
10	21	23·4	0·26	57·1	88·5
20	21	25·3	0·17	67·8	150
30	21	23·3	0·13	81·0	185

Table 6. *Effect of prior equilibration with* 0·1 M-*potassium chloride and* 0·05 M-*sucrose on the uptake of manganese by mitochondria*

The mitochondria were prepared in 0·25 M-sucrose and then added to 0·125 M-KCl solution to give a final concentration in the suspending medium of 0·1 M-KCl and 0·05 M-sucrose. After equilibration for 15 min., $MnCl_2$ was added to separate portions of the suspension to give the concentrations shown below.

Time (min.)	Temp.	Mn^{2+} ions added to medium (μmoles)	Content of Mn^{2+} ions of dry mitochondrial pellet (m-moles/kg.)	Concn. of Mn^{2+} ions in water of		Concn. of Mn^{2+} ions in pellet/concn. of Mn^{2+} ions in medium	Water content of mitochondria (l./kg. dry wt.)
				Pellet (m-molal)	Supernatant (m-molal)		
15	0°	1	24·8	5·8	0·23	25·2	4·28
15 + 11	0 / 24	1	25·8	5·1	0·3	17·0	5·05
15	0	2	33·1	8·4	0·6	14·0	3·93
15 + 11	0 / 24	2	39·8	9·4	0·53	17·7	4·25
15	0	3	43·3	10·4	0·91	11·4	4·17
15 + 11	0 / 24	3	57·5	13·4	0·81	16·6	4·27
15	0	4·2	51·4	12·5	1·49	8·4	4·11
15 + 11	0 / 24	4·2	66·8	17·3	1·3	13·3	3·86
15	0	8·7	66·2	16·5	3·4	4·9	4·00
15 + 11	0 / 24	8·7	93·4	23	3·3	7·0	4·05

also tested (Table 6). The amount of Mn^{2+} ions taken up was very similar to that taken up when the KCl and Mn^{2+} ions were added simultaneously. This shows that potassium and manganese did not compete. However, the mitochondria swelled during the preliminary treatment with KCl, and not all this water was expelled on the addition of Mn^{2+} ions. Thus the concentration gradients were considerably less than when the two salts were added together. This finding suggests that the mitochondrial mass rather than the concentration gradient determines the amount of Mn^{2+} ions taken up. More Mn^{2+} ions were taken up at higher temperatures.

Effect of anaerobiosis on uptake of manganese. The extra uptake of Mn^{2+} ions that occurred in mitochondria incubated at 21° could have been due either to metabolism or to an 'adsorption' process with a high activation energy (chemisorption). Any such chemisorption process is unlikely to be affected by the presence of oxygen. In Fig. 1 is shown the time course of uptake of Mn^{2+} ions by mitochondria aerobically and anaerobically at 25° and aerobically at 0°. The mitochondria at 0° were added to the chilled medium contained in a measuring cylinder which was stoppered, the contents were mixed and it was placed in a bath of ice–water. The mitochondria which were to be

Table 7. *Effect of ethylenediaminetetra-acetic acid on the uptake of manganese by mitochondria*

The mitochondria were prepared and maintained in 0·25 M-sucrose at 0°. Manganese chloride (1·8 μmoles) and the amounts of EDTA given below were added to a series of samples (2 ml.) of the suspension. After 15 min. the samples were centrifuged to separate the mitochondrial pellet for analysis. The water content of the mitochondrial pellet before the addition of Mn^{2+} ions was 2·49 l./kg. of dry mitochondrial pellet.

EDTA added (μmoles)	Content of Mn^{2+} ions of pellet (μmoles)	Concn. of Mn^{2+} ions in water of		Concn. of Mn^{2+} ions in pellet/concn. of Mn^{2+} ions in medium	Water content of pellet (l./kg. dry wt.)
		Pellet (m-molal)	Supernatant (m-molal)		
0	1·15	13	0·37	35·2	2·23
0·4	1·03	11·4	0·42	26·9	2·28
1·0	0·73	8·0	0·55	14·5	2·27
1·94	0·3	3·1	0·72	4·3	2·42
4·0	0·022	0·24	0·93	0·26	2·34
9·6	0·023	0·23	0·86	0·27	2·56

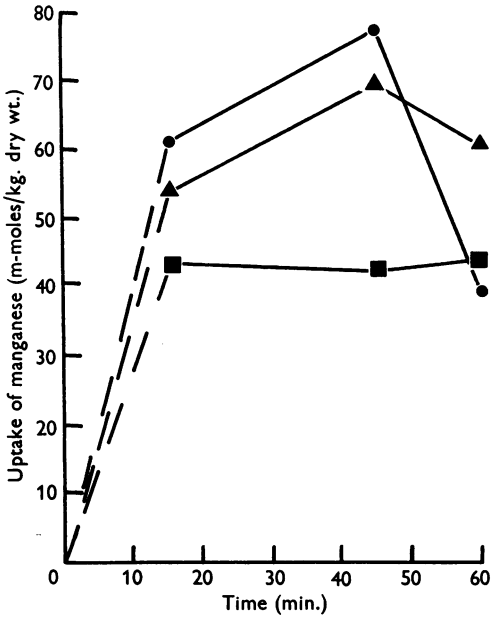

Fig. 1. Effect of anaerobiosis on uptake of manganese by mitochondria. The preparation and incubation of the mitochondria are described in detail in the text. ●, Incubation at 25° in O_2; ▲, incubation at 0° in air; ■, incubation at 25° in N_2.

incubated at 25° were added to Warburg vessels which were filled with O_2 or N_2 at 0°. The mitochondria were tipped from the side arm immediately before putting the manometers in the water bath at 25°. The manometers were removed from the bath at the appropriate times (15, 45 and 60 min.) and a sample (2 ml.) of the vessel contents was centrifuged immediately at high speed to separate the mitochondria from the medium. As shown in Fig. 1, there was a large uptake of Mn^{2+} ions during the first 15 min. under all three conditions, but the amount in N_2 was only about 69% of that in O_2 at the same temperature. Further incubation did not cause an increase in the content of Mn^{2+} ions of the anaerobic mitochondria. In the presence of O_2 there was an increase during the next 30 min. (61–76 m-moles of Mn^{2+} ions/kg. dry wt.) followed by a loss. The mitochondria maintained at 0° showed changes in content of Mn^{2+} ions which were similar to, but less in amount than, those shown by the mitochondria incubated at 25° in O_2.

These observations suggested that whereas part of the uptake of Mn^{2+} ions by mitochondria may be accounted for by an adsorption process there may be an additional uptake by active processes.

Effect of ethylenediaminetetra-acetic acid on uptake of manganese by mitochondria. EDTA is a strong chelator of Mn^{2+} ions and is frequently added to mitochondrial preparations, as it is reported to stabilize enzyme systems (see Slater & Cleland, 1953; Beaufay, Hers, Berthet & de Duve, 1954). When the action of EDTA on uptake of Mn^{2+} ions was studied, it was found that it strongly competed with the mitochondria for the added Mn^{2+} ions (Table 7). A concentration of 0·002 M-EDTA completely inhibited uptake of Mn^{2+} ions. When allowance is made for the interstitial fluid occluded in the mitochondrial pellet (about one-third of the total fluid of the pellet), it can be calculated from the data in Table 7 that about 2·6 μmoles of EDTA were necessary to chelate the 1·8 μmoles of Mn^{2+} ions added to the medium.

Changes of pH *during uptake of manganese by mitochondria.* The pH of mitochondrial suspensions stored in 0·25 M-sucrose or in sucrose–KCl mixtures remains constant at about 7·2. The uptake of Mn^{2+} ions by mitochondria is accompanied by a fall in the pH of the suspension. Table 8 shows that the suspension became more acid with increasing amounts of Mn^{2+} ions added to the suspension. The most probable explanation is that Mn^{2+} ions are bound to a site on the mitochondrial structure by a chelating mechanism which results in the displacement of a H^+ ion. An even larger fall in pH

occurred when mitochondria suspended in sucrose were added to a KCl–$MnCl_2$ mixture (Table 8, Expt. 2). This is probably correlated with the larger uptake of Mn^{2+} ions that occurred in this medium (compare Tables 4 and 5). The change in pH apparently varied with the metabolic state of the mitochondria. Thus the fall in pH was slightly less when the suspension was warmed up in N_2, markedly less when the suspension was warmed in O_2 and was only transitory when substrate was added (Table 8). The reason for this is obscure, as the binding of Mn^{2+} ions is greater when the temperature is raised. Table 9 shows the balance of ion movements that occurred when $MnCl_2$ at different concentrations was added to mitochondria in 0·25 M-sucrose solution. The production of acid was estimated indirectly by titrating a separate sample of mitochondrial suspension with HCl and noting the amount of acid needed to lower the pH to the levels previously observed on addition of $MnCl_2$.

As shown in Table 9, there was an overall loss of cation from the mitochondria. This was probably compensated for by a loss of phosphates (see Amoore & Bartley, 1958). It may be concluded that the uptake of Mn^{2+} ions is mainly compensated for by the release of H^+ ions and that the final balance is achieved by a small uptake of Cl^- ion and the loss of K^+ and Mg^{2+} ions from the mitochondria. It is possible that the movements of the last two ions are secondary effects of the change in pH, for the addition of HCl alone to produce a similar change in pH caused a large loss of K^+ions and some of Mg^{2+} ions, as shown in the last line of Table 9.

Changes in content of potassium chloride of mitochondria on incubation with manganese chloride. When mitochondria in 0·25 M-sucrose solution are added to a KCl–$MnCl_2$ mixture (to give finally 0·1 M-KCl, 0·05 M-sucrose and 0·001 M-$MnCl_2$), Cl^- ion is found at a considerably lower concentration (Table 10) in the mitochondria than in the medium, but when the $MnCl_2$ is added after pre-incubation for 15 min. with KCl, both the Cl^- and K^+ ions freely enter the mitochondria. If the Mn^{2+} ions are bound to the same sites under the two different conditions, it is not at all clear why the movements of Cl^- ions should be so different. Under both conditions prolongation of incubation time at 0° or elevation of the temperature (Table 11) resulted in a net entry of chloride.

Table 8. *Changes in* pH *during uptake of* Mn^{2+} *ions by mitochondria*

In Expt. 1 the mitochondria were prepared and incubated as in Table 1. In Expt. 2, at 25°, the mitochondria were shaken in Warburg vessels and a portion (2 ml.) of the suspension was removed for centrifuging at the times given below.

Expt. no.	Incubation medium	Other conditions	Temp.	Concn. of Mn^{2+} ions (m-molal)	Incubation time (min.)	pH of suspension
1	0·25 M-Sucrose	—	0°	0	—	7·2
	0·25 M-Sucrose	—	0	1	15	6·8
	0·25 M-Sucrose	—	0	2	15	6·6
	0·25 M-Sucrose	—	0	5	15	6·4
2	0·25 M-Sucrose	—	0	0	—	7·2
	0·1 M-KCl +	—	0	1	15	6·1
	0·05 M-sucrose				45	6·0
					60	6·2
	0·1 M-KCl +	Gassed with N	25	1	15	6·2
	0·05 M-sucrose				45	6·3
					60	6·4
	0·1 M-KCl +	Gassed with O_2	25	1	15	6·7
	0·05 M-sucrose				60	6·9
	0·1 M-KCl +	Gassed with O_2 and 200 μmoles	25	1	15	6·9
	0·05 M-sucrose	of sodium succinate added as substrate			45	7·4
					60	7·5

Table 9. *Summary of the ion movements compensating for uptake of manganese*

The mitochondria were prepared and separated as described in Table 1. They were incubated for 15 min. before centrifuging. $\Sigma = (\Delta Mn^{2+} + \Delta K^+ + \Delta Mg^{2+}) - (\Delta Cl^- + \Delta H^+)$, where Δ = change in mitochondrial content of the ion or change of titratable acid in the suspension.

Additions to medium	Change in ion content (Δ) of mitochondrial pellet (m-equiv./kg. dry wt.) Mn^{2+}	Cl^-	K^+	Mg^{2+}	Acid produced (m-equiv./kg. dry wt.) H^+	Σ all ions (m-equiv.)
mM-$MnCl_2$	+50	+3	−15	−12	44	−24
2 mM-$MnCl_2$	+86	+7	−15	−8	69	−13
5 mM-$MnCl_2$	+120	+24	−15	−22	79	−20
mM-HCl		−1	−25	−6	(36)	−30

Table 10. *Effect of manganese on potassium and chloride content of mitochondria at* 0°

In Expts. 1, 2 and 4, the mitochondria were prepared in 0·25 M-sucrose and added to a mixture of KCl and $MnCl_2$ to give the concentrations stated below. In Expt. 3, the Mn^{2+} ions were added after the mitochondria had equilibrated with 0·1 M-KCl and 0·05 M-sucrose for 15 min. The excess of each ion is the amount remaining, after deduction from the total, of the amount that would be in solution in the mitochondrial water if the ion were at the same concentration as in the external medium.

Expt. no.	Time (min.)	Medium	Concn. of Mn^{2+} ions (m-molal)	Excess of K^+ ions (m-moles/kg. of dry mitochondrial pellet)	Excess of Cl^- ions (m-moles/kg. of dry mitochondrial pellet)
1	11·5	0·1 M-KCl + 0·05 M-sucrose	1	0	−68
	60		1	0	−49
2	10	0·1 M-KCl + 0·05 M-sucrose	1	−10	−60
3	0	0·1 M-KCl + 0·05 M-sucrose	0	−5	−54
	15		0	16	−5
	15		1	13	53
	50		1	42	71
4	15	0·25 M-Sucrose	1	35	7·8

Table 11. *Effect of manganese on potassium and chloride content of mitochondria on incubation*

In Expt. 1 the mitochondria were prepared in 0·25 M-sucrose and added to a mixture of KCl and $MnCl_2$, as in Table 10 (Expts. 1 and 2), and in Expt. 2 the Mn^{2+} ions were added after equilibration with 0·1 M-KCl and 0·05 M-sucrose for 15 min. Concn. of Mn^{2+} ions was mM.

Expt. no.	Time (min.)	Temp.	Excess of K^+ ions (m-moles/kg. of dry mitochondrial pellet)	Excess of Cl^- ions (m-moles/kg. of dry mitochondrial pellet)
1	11·5	20°	4	−57
	60	20	28	−45
2	11	23·8	52	95
	22·5	23·8	46	62

Table 12. *Effect of manganese on the magnesium content of mitochondria*

The mitochondria, prepared in 0·25 M-sucrose, contained 30·3 m-moles of Mg^{2+} ions/kg. At zero time the mitochondria, suspended in 0·25 M-sucrose, were added to KCl soln. to give a final concn. in the medium of 0·1 M-KCl and 0·05 M-sucrose. After 20 min., 9 ml. of the suspension was added to tubes containing varying amounts of $MnCl_2$. At 35 min. half of the contents of each tube was poured into beakers of 15 ml. capacity and shaken in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago, Ill., U.S.A.) at 24°. Samples were centrifuged for analysis at the times given below.

Concn. of Mn^{2+} ions in medium (mM) ...		0	0·5	1	1·5	2·0	5·0	0	0·5	1·0	1·5	2·0	5·0
Time (min.)	Temp.	Content of Mn^{2+} ions of mitochondria (m-moles/kg. dry wt.)						Content of Mg^{2+} ions of mitochondria (m-moles/kg. dry wt.)					
15	0°	0	—	—	—	—	—	14·6	—	—	—	—	—
35	0	0	24·8	33·1	43·1	51·4	66·2	8·2	9·9	10·9	12·5	15·3	9·8
46	24	0	25·8	39·8	57·5	66·8	93·4	9·3	6·4	7·0	10·3	6·5	6·1
57·5	24	0	16·6	36·1	52·5	87·0	82·6	9·4	6·3	7·5	14·4	7·4	6·0
70	0	0	21·9	32·5	41·2	53·3	77·1	9·1	9·7	9·1	10·0	9·6	10·0

Effect of manganese on the content of Mg^{2+} *and* Ca^{2+} *ions of the mitochondria.* Several experiments (when the unreliable Titan yellow method was still used) indicated that the usual content (30–35 m-moles/kg.) of Mg^{2+} ions of mitochondria prepared in 0·25 M-sucrose was reduced to somewhat less than half (see also Siekevitz & Potter, 1955) on adding the particles to a KCl–$MnCl_2$ mixture (final concentrations: 0·1 M-KCl, 0·05 M-sucrose, 0·001 M-$MnCl_2$). Subsequently, these experiments were repeated by adding the mitochondria first to KCl solution and then, after about 15 min., to the $MnCl_2$ solution. The estimations were done with the modification of the method of Mann & Yoe (1956) described under Methods, with the use of 8-hydroxyquinoline to extract the Mn^{2+} ions. Table 12 shows that in this mitochondrial preparation most of the endogenous Mg^{2+} ions were lost

Table 13. *Effect of ethylenediaminetetra-acetic acid on the uptake of manganese by mitochondria and on their content of* Mg^{2+} *and* Ca^{2+} *ions*

The mitochondria were prepared and incubated as in Table 1, except that the amounts of EDTA given below were added to the incubation medium. Incubation time, 5 min. at 0°.

Concn. of EDTA in medium (m-molal)	Concn. of Mn^{2+} ions in medium (m-molal)	Content of Mn^{2+} ions of dry mitochondrial pellet (m-moles/kg.)	Content of Mg^{2+} ions of dry mitochondrial pellet (m-moles/kg.)	Content of Ca^{2+} ions of dry mitochondrial pellet (m-moles/kg.)
0	0	0	11·2	31·5
0	1	29	6·8	13·7
0·2	1	26·1	8·6	14·5
0·5	1	18·4	7·6	16·0
0·97	1	7·5	11·5	14·0
2·0	1	0·6	7·8	6·7
4·8	1	0·6	8·2	5·4

during the first 15 min. after the addition of the KCl solution at 0°; it fell to a minimum by 35 min. and thereafter there was a slight uptake of the Mg^{2+} ions, which were retained for a further 50 min. The addition of Mn^{2+} ions, 15 min. after the KCl, inhibited the loss of Mg^{2+} ions. All concentrations of Mn^{2+} ions were not equally effective in preventing the loss of Mg^{2+} ions, the most effective being about 1·5 mM. On the other hand, at 24° Mn^{2+} ions caused an increase in the loss of Mg^{2+} ions except at the optimum concentration of Mn^{2+} ions (1·5 mM), where there was an uptake of Mg^{2+} ions with time. By contrast, as shown by Table 11, Mn^{2+} ions causes a loss of Mg^{2+} ions from mitochondria in sucrose, and this was about equal to the extra quantity of Mg^{2+} ions that was displaced by Mn^{2+} ions at 24° subsequent to treatment with KCl. Even when the level of endogenous Mg^{2+} ions of the mitochondria was low (Table 13), Mn^{2+} ions still caused the same displacement of Mg^{2+} ions from mitochondria suspended in 0·25 M-sucrose.

The calcium content of our mitochondrial preparations is fairly constant at between 25 and 30 m-moles/kg. of dry mitochondria. This calcium was not displaced when the mitochondria were suspended in a mixture of 0·1 M-KCl, 0·05 M-sucrose and mM-$MnCl_2$. It was, however, displaced when mM-$MnCl_2$ was added to a mitochondrial suspension in 0·25 M-sucrose (Table 13, compare lines 1 and 2). It is of interest that 0·2 mM-EDTA displaced an almost identical amount of calcium from the same preparation of mitochondria as did the mM-$MnCl_2$. The addition of both 0·2 mM-EDTA and mM-$MnCl_2$ did not result in an additive effect. Further calcium was displaced when sufficient EDTA had been added to chelate all the added manganese (Table 13, lines 6 and 7). It appears probable from these observations that the calcium, which is easily displaced from the mitochondria, is located on the outer mitochondrial surface.

Effect of manganese on the leakage of phosphate compounds from the mitochondria at 0°. An earlier section of this paper has shown that nucleotide-like substances leak from the mitochondria on storage at 0° and that this may be prevented in some circumstances by the addition of Mn^{2+} ions. Also, Amoore & Bartley (1958) have shown a progressive leakage of phosphate esters from rat-liver mitochondria stored at 0° in either 0·25 M-sucrose or in 0·125 M-KCl. Table 14 shows that leakage of phosphate from the mitochondria was practically abolished by the addition of 1·5 mM-$MnCl_2$ to the medium.

Table 14. *Effect of manganese on the leakage of phosphate from mitochondria at* 0°

The mitochondria were prepared in 0·25 M-sucrose and then added to 0·125 M-KCl to give a final concentration in the suspending medium of 0·1 M-KCl and 0·05 M-sucrose. After equilibration for 15 min. at 0°, $MnCl_2$ was added to separate portions of the suspension to give the concentrations stated below. The mitochondria initially contained 83·1 m-moles of phosphate/kg. dry wt.

Time (min.)	Incubation medium	Excess of phosphate content (m-moles/kg. of dry mitochondria)
0	0·1 M-KCl + 0·05 M-sucrose	73·6
20		67·6
35		50·0
70		49·0
15	0·1 M-KCl + 0·05 M-sucrose + 0·5 mM-$MnCl_2$	61·1
50		57·8
15	0·1 M-KCl + 0·05 M-sucrose + mM-$MnCl_2$	61·6
50		58·3
15	0·1 M-KCl + 0·05 M-sucrose + 1·5 mM-$MnCl_2$	66·8
50		65·8
15	0·1 M-KCl + 0·05 M-sucrose + 5 mM-$MnCl_2$	58·4
50		57·3

Effect of manganese on the leakage of phosphate compounds from the mitochondria at 24°. As shown in Table 15, raising the temperature to 24° resulted

in a greater loss of phosphate from mitochondria than occurred at 0°. This is consistent with the observation often made that mitochondria become metabolically inactive more rapidly at elevated temperatures than at 0°. Table 15 also shows that even 0·5 mM-$MnCl_2$ slowed the rate of phosphate loss. When the $MnCl_2$ concentration added was mM, not only was the loss of phosphate inhibited, but there was an uptake of the endogenous phosphate that had previously leaked from the mitochondria. With 1·5 mM-$MnCl_2$ the content of phosphate of the mitochondria was restored to the original concentration found when the particles were first isolated in sucrose solution. As at 0°, 5 mM-$MnCl_2$ was not so effective as 1·5 mM-$MnCl_2$ in preventing loss of phosphate or in causing uptake of phosphate. Continued incubation of the mitochondria in the presence of Mn^{2+} ions at 24° resulted in a loss of phosphate. This loss was minimal when the $MnCl_2$ was added at 1·5 mM concentration. It appears probable that both the extra uptake of Mn^{2+} ions and of phosphate that occurred on raising the temperature from 0° to 24° were the result of active processes.

Table 15. *Effect of manganese on the leakage of phosphate from mitochondria at* 24°

The mitochondria were those of Table 14. At time 20 min. in Table 14 the mitochondria were warmed to 24° for the times stated below. The mitochondria initially contained 83·1 m-moles of phosphate/kg. dry wt.

Time (min.)	Incubation medium	Excess of phosphate content (m-moles/kg. of dry mitochondria)
11	0·1 M-KCl + 0·05 M-sucrose	31·6
22·5		38·5
11	0·1 M-KCl + 0·05 M-sucrose + 0·5 mM-$MnCl_2$	46·4
22·5		39·2
11	0·1 M-KCl + 0·05 M-sucrose + 1 mM-$MnCl_2$	80·6
22·5		74·1
11	0·1 M-KCl + 0·05 M-sucrose + 1·5 mM-$MnCl_2$	86·1
22·5		84·2
11	0·1 M-KCl + 0·05 M-sucrose + 5 mM-$MnCl_2$	80·4
22·5		75·3

Manganese and mitochondrial swelling. Mitochondria sometimes swell in isotonic solutions and often swell in hypotonic solutions, the effect depending on the composition of the medium. The reason for the swelling in isotonic solutions is not known and in our experience the phenomenon is unpredictable. It is, however, more likely to occur when the temperature of the suspending medium is raised above 0°. The Mn^{2+} ion is able to prevent or slow down the rate of swelling of mitochondria over a wide range of conditions (see Fonnesu & Davies, 1956; Tapley, 1956). In our experiments manganese always caused a shrinkage in the mitochondria at 0° in 0·25 M-sucrose solution (Tables 1–4 and 16). Increasing concentration of

Table 16. *Effect of manganese on the water content of mitochondria at* 0°

Expt. no.	Time (min.)	Suspending medium	Initial content of Mn^{2+} ions of medium (mM)	Water content of mitochondria: Without Mn^{2+} ions (l./kg. dry wt.)	Water content of mitochondria: With Mn^{2+} ions (l./kg. dry wt.)
1	—	0·25 M-Sucrose	0	1·93	—
	0	0·1 M-KCl + 0·05 M-sucrose	0	2·39	—
	0		1	—	2·18
	60		0	2·50	—
	60		1	—	2·32
2	—	0·25 M-Sucrose	0	2·25	—
	2	0·1 M-KCl + 0·05 M-sucrose	1	—	2·10
3	—	0·25 M-Sucrose	0	2·27	—
	5	0·1 M-KCl + 0·05 M-sucrose	1	—	2·96
4	0	0·25 M-Sucrose	0	2·49	—
	0	0·1 M-KCl + 0·05 M-sucrose	1	—	2·23
5	—	0·25 M-Sucrose	0	2·48	—
	0	0·1 M-KCl + 0·05 M-sucrose	0	3·89	—
	15		0	4·2	—
	50		0	4·36	—
	15		0·5	—	4·28
	50		0·5	—	4·77
	15		1·0	—	3·93
	50		1·0	—	3·95
	15		1·5	—	4·17
	50		1·5	—	4·31
	15		5·0	—	4·00
	50		5·0	—	4·16

Mn^{2+} ions in the medium (Table 4) increased the loss of mitochondrial water, and no water was lost when sufficient EDTA was added to the medium to chelate all the Mn^{2+} ions (Table 7). On raising the temperature to 20–25° the mitochondria swelled, whether Mn^{2+} ions were present or not, but the swelling was less when Mn^{2+} ions were present (Tables 1, 2 and 17). In saline media the findings are not so consistent. Usually when mitochondria in sucrose solution are transferred to a saline medium of equivalent osmotic pressure at 0°, there is a slight swelling (Table 16). If the saline medium also contains Mn^{2+} ions, the swelling is usually less and on occasions the mitochondria shrink to a water content less than that of the mitochondria in sucrose solution (Table 16, Expt. 2); compare also Fonnesu & Davies (1956). When the Mn^{2+} ions were added after equilibration with 0·1 M-KCl, swelling was still prevented, but there was no simple relationship between the amount of Mn^{2+} ions added and the inhibition of swelling.

DISCUSSION

The findings reported in this paper clearly show that metabolic behaviour and permeability of mitochondria in solutions which are mainly non-ionic are different from those of mitochondria in largely ionic media. In general, mitochondria appear to be somewhat more permeable in ionic solutions and more liable to loss of endogenous solutes, for example, magnesium and phosphate compounds. The addition of manganese to the suspending medium in optimum amounts appears to delay or reverse this 'ageing' process. The manganese is more effective in this role in the non-ionic media as judged by the rate at which E_{260} substances leak from the mitochondria. The data are consistent with the idea that saline solutions produce an increase in the permeability of the mitochondrial membrane to anions. Manganese in some way tends to prevent this increase in permeability. This is best illustrated by considering the results on the distribution of chloride. In sucrose solutions the uptake of manganese is not matched by an equivalent uptake of chloride and a shrinkage of the mitochondria occurs. A similar phenomenon occurs when magnesium chloride or hydrochloric acid is used instead of manganese chloride. All these observations are consistent with a relatively slow permeation of the mitochondrial membrane by the Cl^- ion. After treatment with potassium chloride solution under the conditions described in Table 10 and the subsequent addition of manganese chloride, the uptake of manganese and chloride is more or less equivalent and no shrinkage occurs. The swelling of mitochondria exposed to the action of the salt solutions alone is reduced by the addition of Mn^{2+} ions. In one case (Table 16, Expt. 2) there was actually a shrinkage in the mitochondria compared with those suspended in 0·25 M-sucrose. Once again chloride was found at a lower concentration in the pellet than in the medium. This argument implies that the shrinkage of mitochondria in the presence of manganese is a secondary effect due to the impermeability of the mitochondrial membrane to the external chloride.

It is considered unlikely that the manganese taken up by the mitochondria is in free solution and is osmotically active, because the intramitochondrial fluid would then be grossly hypertonic and the mitochondria would swell. It is much more likely that the manganese is bound to the mitochondria by some form of adsorption. It is suggested that the amount of manganese taken up reflects the amount of mitochondrial protein accessible to the manganese. Thus the initial rapid uptake that occurs at 0° is probably adsorption upon the mitochondrial membrane, whereas the slower uptake with time represents a slow penetration of the manganese to sites within the mitochondria. The greater uptake of manganese occurring in mainly ionic media represents the greater permeability of the mitochondrial membrane that occurs in these media. It is clear from the work of Stanbury & Mudge (1953) on the exchange of potassium in mitochondria, and from the observations on loss of nucleotide given in this paper, that the permeability of the mitochondrial membrane is increased during metabolism. Thus it might be expected that the uptake of manganese would also be increased by increase of temperature, and hence of metabolism, by the increased accessibility to binding sites

Table 17. *Effect of manganese on the water content of mitochondria incubated at 24°*

The incubation medium was 0·1 M-KCl + 0·05 M-sucrose. The incubation conditions were as described in Table 3.

Expt. no.	Time (min.)	Initial content of Mn^{2+} ions of medium (mM)	Water content of mitochondria: Without Mn^{2+} ions (l./kg. dry wt.)	Water content of mitochondria: With Mn^{2+} ions (l./kg. dry wt.)
1	15	0	2·25	—
	15	1	—	2·31
	30	0	3·15	—
2	11	0	5·6	—
	22·5	0	6·3	—
	11	0·5	—	5·05
	22·5	0·5	—	5·39
	11	1·0	—	4·25
	22·5	1·0	—	4·43
	11	1·5	—	4·27
	22·5	1·5	—	4·24
	11	5·0	—	4·05
	22·5	5·0	—	4·17

within the mitochondria. Two observations argue against this simple explanation of uptake of manganese. The first is the difference occurring aerobically and anaerobically, and the second the loss of manganese that occurred during the later stages of incubation at 25°. It is feasible that the mitochondria may not be so permeable under anaerobic conditions as they are aerobically, and this may, in turn, reflect the difference in metabolism under the two conditions. The loss of manganese occurring during prolonged incubation may merely be a measure of protein leakage from the mitochondria. Any other explanation would seem to involve a more direct coupling of metabolism with uptake of manganese ('active transport') and this cannot at present be excluded.

It seems unlikely that the effects of manganese recorded in this paper can apply to the mitochondria *in vivo*, as the total liver content of manganese, even if all were concentrated in the mitochondria, amounts to only about 1–5 % of the amount taken up by the mitochondria in mM-manganese chloride, although even at such low levels the amount of Mn^{2+} ions present may condition the permeability of the mitochondrial membrane.

Whatever the means whereby manganese functions in the stabilization of the mitochondrial membrane, it appears that its uptake is dependent on the presence of free Mn^{2+} ions, for the uptake was completely abolished by the addition of an equivalent amount of ethylenediaminetetra-acetic acid.

Although most of the observations on movements of ions resulting from the addition of manganese chloride to a mitochondrial suspension can be explained as the consequence of a change in permeability of the mitochondrial membrane, it is not easy to explain the recovery of endogenous phosphate and magnesium which occurred on incubation of the mitochondria with 1·5 mM-manganese chloride. It is difficult to escape the conclusion here that Mn^{2+} ions are closely associated with the active uptake process.

The large uptake of manganese by mitochondria that has been demonstrated emphasizes the danger in assuming that in cell-free preparations the added solutes are evenly distributed throughout the suspension. The possibility of such localization of solutes needs to be borne in mind when deducing the probable direction of reaction sequences from thermodynamic data and overall concentrations of reactants.

SUMMARY

1. At 20°, mM-manganese chloride decreased the loss of ultraviolet-light-absorbing nucleotide-like substances and protein from mitochondria suspended in 0·25 M-sucrose. Manganese did not affect nucleotide loss when the suspending medium was 0·1 M-potassium chloride plus 0·05 M-sucrose. In either medium, mM-manganese chloride maintained the mitochondria at a higher dry weight than in its absence.

2. Mitochondria suspended in 0·25 M-sucrose and mM-manganese chloride took up manganese at a high rate at 0°, reaching about 25 m-moles/kg. dry weight of mitochondria. The uptake was increased when the concentration of manganese chloride in the medium was increased, but not proportionally. With 5 mM-manganese chloride the uptake reached 60 m-moles/kg. dry weight. The loss of water from the mitochondria was higher in the presence of 5 mM-manganese chloride than with mM.

3. The uptake of manganese by mitochondria suspended in 0·1 M-potassium chloride, 0·05 M-sucrose and mM-manganese chloride at 0° was about 1·5 times as fast as in 0·25 M-sucrose. A further increase of 1·5 times in the rate of uptake occurred when the temperature was raised to 20°. The total uptake reached 81 m-moles/kg. dry weight. The uptake of manganese was about the same when the manganese chloride was added after the mitochondria had equilibrated with the potassium chloride–sucrose mixture.

4. After incubation for 15 min. in nitrogen at 25°, the uptake of manganese of mitochondria suspended in 0·1 M-potassium chloride plus 0·05 M-sucrose was only 69 % of the uptake in oxygen. Further incubation caused an increase of the manganese content of the mitochondria in oxygen but not of those in nitrogen.

5. The uptake of manganese by mitochondria suspended in 0·25 M-sucrose and mM-manganese chloride was completely inhibited by 1·5 mM-ethylenediaminetetra-acetic acid.

6. The addition of manganese chloride to a mitochondrial suspension caused a fall in the pH of the suspension of as much as 1 pH unit. The change of pH was increased by an increase of manganese concentration and by the presence of potassium chloride in the medium and decreased by raising the temperature above 0°. In 0·25 M-sucrose solution, between 60 and 90 % of the uptake of manganese could be accounted for by the liberation of H^+ ions.

7. Manganese maintained the impermeability of the mitochondrial membrane to chloride.

8. At 0°, mM-manganese chloride displaced about one-third of the endogenous magnesium content and about one-half of the endogenous calcium content. Transfer of mitochondria from 0·25 M-sucrose to 0·1 M-potassium chloride plus 0·05 M-sucrose caused a loss of about half the endogenous magnesium, but no loss of the endogenous calcium. Manganese prevented the loss of

magnesium at 0° which occurs in the salt–sucrose mixture. The most effective concentration was 1·5 mM. Incubation of mitochondria at 24° in 0·1 M-potassium chloride, 0·05 M-sucrose and 1·5 mM-manganese chloride after they had lost endogenous magnesium at 0°, resulted in an uptake of most of the magnesium lost.

9. The loss of mitochondrial phosphate that occurred in 0·1 M-potassium chloride plus 0·05 M-sucrose at 0° was abolished by the addition of 1·5 mM-manganese chloride to the medium. Incubation at 24°, in the manganese-containing medium, of mitochondria which had already lost phosphate resulted in a restoration of the phosphate content.

10. In sucrose or saline media at 0° or at 25° the presence of mM-manganese chloride resulted in a lower water content of mitochondria than occurred in its absence.

11. The mode of action of manganese in the stabilization of mitochondria is discussed.

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The Esterase Activity of the Fibrinolytic System

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The solubilization of fibrin clots in mammalian blood is caused by a proteolytic enzyme termed plasmin. This enzyme is not present in significant concentrations in normal plasma, but it can be produced from its precursor, plasminogen, through the interaction of appropriate activating substances.

The most potent and commonly used activator of human plasminogen preparations is streptokinase. This substance is found in the culture filtrate from certain strains of β-haemolytic streptococci (Tillett & Garner, 1933). Investigations by Kaplan (1944) and Christensen (1945) revealed the activating properties of the streptococcal product. The conclusion that the conversion of plasminogen into plasmin is catalysed by streptokinase was reached by Christensen & McLeod (1945), and although the reaction has later proved to be more complicated (Geiger, 1952; Müllertz & Lassen, 1953; Sherry, 1954; Asahina & Oka, 1954), it is still probable that the last step in the reaction sequence, the conversion of plasminogen into plasmin, is enzymic.

Müllertz & Lassen (1953) suggested that streptokinase is unable to activate pure human plasminogen, but that an activator of plasminogen is formed through a reaction between streptokinase and a substance, termed proactivator, which is present in large amounts in human plasma. This concept was accepted by Troll & Sherry (1955), but was later rejected by Sherry, who now presumes identity between plasminogen and proactivator and supposes the activator to be a complex of streptokinase with plasminogen or plasmin (Sherry & Alkjaersig, 1956).

It seems to be commonly accepted that the formation of activator proceeds through a stoicheiometric reversible reaction between streptokinase