Differences in Sodium and Potassium Loss by Kidney Mitochondria

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In a study of ion transport by rat-heart and skeletal-muscle mitochondria, Ulrich (1959) presented evidence that the potassium, but not the sodium, gradient maintained by these particles could be increased by metabolic activity during incubation at 37°. Bartley & Davies (1954) reported that sheep-kidney mitochondria transported both of these ions against concentration gradients when adenosine triphosphate and an oxidizable substrate were present in the incubating medium. Since it was of some interest to determine the cause for these differences, some of the experiments of Bartley & Davies were repeated with rabbit-kidney mitochondria. Their findings with respect to sodium transport could not be confirmed. The results demonstrate that the sodium gradient maintained by rabbit-kidney mitochondria cannot be increased by metabolic activity, and that in several other respects the transport of these two ions by kidney mitochondria is different. A preliminary account has been given (Ulrich, 1961).

METHODS

Chemicals. Adenosine triphosphate (disodium or potassium salt; ATP) was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. Adenosine 5'-phosphate (sodium salt; AMP) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. α -Oxoglutaric acid (A grade) was obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Succinic acid (Fisher certified reagent) and fumaric acid (Fisher purified) were obtained from the Fisher Scientific Laboratory, New York. L-Glutamic acid was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. 2,4-Dinitrophenol was obtained from the Eastman Kodak Co., Rochester, New York. Sucrose was analytical grade.

Preparation of mitochondria. Mitochondria were isolated from the kidneys of white, New Zealand strain, male rabbits (about 3 kg. body wt.). All operations were carried out at 2–4°. The animals were stunned by a blow at the back of the neck and the kidneys were rapidly removed and plunged into 0.25 M-sucrose solution at 0°, which was prepared not more than 18 hr. before the beginning of the experiment. The capsule was removed and, after each kidney had been sagittally divided into halves, the kidneys were transferred twice to fresh 0.25 M-sucrose solutions, blotted on filter paper to remove excess of sucrose and then minced with scissors. Portions (5 g.) of mince were homo-

genized with a size C Teflon model homogenizer (Arthur H. Thomas Co.) for approx. 1 min. (17 complete strokes of the homogenizer) with 35 ml. of 0.25 M-sucrose. Sucrose (10 ml.) was then added to give a 10% (w/v) homogenate. The homogenate was centrifuged at 600g in a PR-2 International refrigerated centrifuge at 0° for 5 min. The resulting supernatant was filtered through two layers of water-washed no. 120 Curity-grade cheese-cloth (Kendall Co., Walpole, Mass.) and then centrifuged for 10 min. at 7600 rev./min. (5000g) in the no. 30 rotor of the Spinco model L preparative ultracentrifuge. The mitochondria were washed twice with approx. 3 ml. of 0.25 M-sucrose/g. of mince, first by resuspending the mitochondria with a homogenizer and centrifuging for 10 min. at approx. 16 700 rev./min. (25 000g), and secondly by resuspending with a stirring rod and again centrifuging at 25 000g for 10 min. The surface of the mitochondrial pellets was also rinsed after each centrifuging with small amounts of the sucrose solution to remove as much of the fluffy layer as possible. After the second washing, the centrifuge tubes were drained, the inside walls wiped with filter paper and the mitochondria immediately suspended in the appropriate solution.

Solutions. All solutions were prepared with water redistilled with Pyrex glassware. Solutions containing an adenine nucleotide and oxidizable substrate were adjusted to pH 7.4 with solid tris (2-amino-2-hydroxymethylpropane-1,3-diol). The same amount of tris was added to the control solution and the pH of this solution was then adjusted to 7.4 with 5N- and 1N-HCl. A Beckman pH meter, model G, with glass electrode, standardized with Beckman buffer solution, pH 7, was used for all pH measurements.

Nomenclature. Suspending media refer to the solutions with which the mitochondria were suspended. Incubating media refer to the solutions to which the mitochondrial suspensions were added immediately before starting the incubation. Adenine nucleotides, oxidizable substrates and inhibitors were added only to the incubating media.

Incubation of mitochondria. In experiments with the 8–10 medium (see next paragraph), 2 ml. of the mitochondrial suspension at 0° were added to 2 ml. of incubating medium in a 20 ml. Pyrex beaker, the incubating medium having first been warmed to 37°. In experiments with the 0–1 medium (see next paragraph), 0.5 ml. of the suspension was added to 3.5 ml. of incubating medium. The 4 ml. suspensions were incubated with gentle shaking in air at 37° in a Dubnoff metabolic incubator. Isolation of the mitochondria after incubation, determinations of mitochondrial wet and dry weights and analyses of sodium and potassium were carried out as described previously (Ulrich, 1959).

Changes in sodium and potassium gradients. Two kinds of experiments were performed: (a) Mitochondria were sus-

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pended in a solution containing 8–10 mM-sodium or potassium or both. The suspension was then added to a solution containing approximately the same amount of sodium and potassium and incubated at 37°. These are referred to below as experiments with the 8–10 medium. (b) The mitochondrial suspension was added to a solution containing either no sodium and potassium or else only very small amounts (approx. 1 mM) of these ions. These are referred to below as experiments with the 0–1 medium. Experiments were performed with the 0–1 medium to obtain considerably steeper sodium and potassium gradients than would have been possible with the 8–10 medium.

In the present study, the mitochondrial sodium and potassium content was measured only at the end of an incubation period. Therefore changes in the ion contents and in the ion gradients maintained by the particles are undoubtedly due to changes in the rate of loss of sodium and potassium from the mitochondria. Experiments (unpublished) have shown that if twice-washed rat-liver mitochondria are suspended in a solution at 0° containing 8–10 mM-potassium, the potassium gradient maintained by the particles is between 7 and 8. However, if the ice-cold suspension is added to medium at 37° with the same potassium concentration, and incubated for 20 min., the potassium gradient decreases to 2.6. Addition of ATP and succinate to the incubating medium increases the gradient to 4.6. Further, the results in Table 2 indicate that as the period of incubation is increased, increasing amounts of potassium and, to a less extent, sodium leak out of the particles. These experiments indicate that the mitochondria are not in a steady state with regard to their sodium or potassium contents when incubated at 37°.

RESULTS

Experiments with the 8–10 medium. Table 1 shows results from experiments with two different mitochondrial preparations in which ATP and a

number of oxidizable substrates were added to the incubating medium. In the first experiment, unwashed mitochondria incubated for 20 min. without ATP and substrate maintained lower sodium and potassium gradients than did twice-washed mitochondria. This appeared to be due to the greater swelling of the unwashed mitochondria, since all data for sodium and potassium were calculated from the mitochondrial water content, i.e. the difference between the wet and dry weight. Addition of ATP and α -oxoglutarate to the medium significantly increased the concentration gradient of potassium but had little effect on the sodium gradient. Similar results were obtained in the second experiment, in which succinate or fumarate was used. The sodium gradient was decreased with ATP and substrate, but this change was small.

Werkheiser & Bartley (1957) have shown that at 0° the percentage dry weight of mitochondria prepared with 0.25M-sucrose at pH 5–6 was significantly less than that of particles prepared with sucrose at pH 7.8. They stated that examination of the pH 5–6 sucrose mitochondria in the phase-contrast microscope showed the particles to be swollen, whereas mitochondria prepared with the pH 7.8 sucrose appeared unswollen. Pauly, Packer & Schwan (1960) reported that rat-liver mitochondria isolated with the usual acid sucrose solution appeared small, dark and shrunken in the phase-contrast microscope. Since our inability to demonstrate an increase in the sodium gradient might have been due to the use of swollen (and thus damaged?) mitochondria, the mitochondria were isolated with the alkaline sucrose solution. The sucrose solution, adjusted to pH 7.8 with 0.2M-tris,

Table 1. *Effects of adenosine triphosphate and substrates on the loss of sodium and potassium from rabbit-kidney mitochondria*

Expt. 1	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of	Sodium in mitochondria (m-equiv./l.)	Ratio of
			potassium contents ($\frac{\text{mitochondria}}{\text{medium}}$)		sodium contents ($\frac{\text{mitochondria}}{\text{medium}}$)
Unwashed control	19.2 ± 0.15	12.7 ± 0.3	1.68 ± 0.05	14.7 ± 0.8	1.69 ± 0.13
Unwashed, ATP + α -oxoglutarate	20.3 ± 0.1	19.2 ± 1.1	2.29 ± 0.14	16.5 ± 1.2	1.57 ± 0.10
Control	28.8 ± 0.0	16.2 ± 0.4	2.19 ± 0.09	17.1 ± 0.7	2.15 ± 0.13
ATP + α -oxoglutarate	28.4 ± 0.2	25.2 ± 0.8	2.89 ± 0.11	21.6 ± 2.0	2.36 ± 0.64
Expt. 2					
Control	25.3 ± 0.1	13.7 ± 0.2	1.65 ± 0.02	15.7 ± 0.2	1.94 ± 0.02
ATP + succinate	24.2 ± 0.2	26.2 ± 0	2.82 ± 0	17.1 ± 0.5	1.73 ± 0.05
Control	24.1 ± 0.3	14.6 ± 0.1	1.76 ± 0.01	16.2 ± 0.1	2.00 ± 0.01
ATP + fumarate	24.9 ± 0.4	27.0 ± 0.1	2.90 ± 0.01	16.9 ± 0.1	1.70 ± 0.03

Unless otherwise indicated, in this and subsequent Tables mitochondria were washed twice with 0.25M-sucrose as described in the text. All figures are the means of three incubations $\pm$ s.e. Where only two incubations were carried out, the figures for both are presented. The mitochondria were suspended with the following solution: 5 mM- $\text{Na}_2\text{H}_2\text{PO}_4$, H_2O - Na_2HPO_4 (pH 7.4), 5 mM- KH_2PO_4 - K_2HPO_4 (pH 7.4), 0.25M-sucrose, 5 mM- MgSO_4 . A portion (2 ml.) of the mitochondrial suspension was added to 2 ml. of incubating medium with the same composition together with or without 4 mM-substrate, 1 mM-sodium ATP and 1 mM-potassium ATP. The mitochondria were incubated in air for 20 min. at 37°.

was used approx. 18 hr. after preparation, at which time the pH had fallen to 7.3. The results (Table 2) show that the loss of sodium from the mitochondria was not consistently or significantly changed with the addition of ATP and α -oxoglutarate to the incubating medium. As the incubation time was increased from 5 min. to 1 hr., the control potassium gradients decreased, until at the end of 1 hr. the gradient was approximately half the 5 min. value. The control sodium gradients decreased only 16% after 1 hr.

The effect of ATP and α -oxoglutarate in increasing the percentage dry weight of the mitochondrial

pellets was least after 5 min. incubation and greatest after 60 min. Although the amount of fluffy layer appeared to be somewhat less with the alkaline sucrose, the movements of sodium and potassium and the percentage dry weight of the particles were similar to those of mitochondria prepared with the acid sucrose. Comparison with Werkheiser & Bartley's results cannot be made because in our experiments mitochondrial water was determined only after a period of incubation at 37° instead of at 0°. Further, in the present experiments the media contained not only sucrose but other compounds as well.

Table 2. *Loss of sodium and potassium from rabbit-kidney mitochondria isolated in an alkaline sucrose solution*

Conditions were the same as described in Table 1 except that the mitochondria were isolated in 0.25 M-sucrose, pH 7.3.

Incubation time (min.)	Additions	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents (mitochondria/medium)	Sodium in mitochondria (m-equiv./l.)	Ratio of sodium contents (mitochondria/medium)
5	None	25.4 ± 0.4	25.7 ± 0.2	3.09 ± 0.025	17.2 ± 0.8	2.07 ± 0.10
5	ATP + α -oxoglutarate	25.7 ± 0.15	33.7 ± 0.2	3.83 ± 0.025	18.0 ± 0.3	1.89 ± 0.04
10	None	26.8 ± 0.2	22.4 ± 0.3	2.80 ± 0.08	17.3 ± 0.5	2.08 ± 0.06
10	ATP + α -oxoglutarate	27.2 ± 0.1	32.7 ± 0.3	3.71 ± 0.03	17.6 ± 0.6	1.86 ± 0.06
20	None	24.0 ± 0.9	15.3 ± 0.4	1.84 ± 0.05	14.0 ± 0.6	1.69 ± 0.08
20	ATP + α -oxoglutarate	26.4 ± 0.1	28.9 ± 0.2	3.29 ± 0.03	17.4 ± 0.1	1.83 ± 0.01
60	None	24.6 ± 0.1	13.6 ± 0.1	1.64 ± 0.01	14.4 ± 0.1	1.73 ± 0.01
60	ATP + α -oxoglutarate	28.1 ± 0.1	20.7 ± 0.2	2.36 ± 0.03	16.8 ± 0.3	1.77 ± 0.03

Table 3. *Effects of adenosine triphosphate and substrates on sodium, potassium and water contents of cortical and medullary rabbit-kidney mitochondria*

Conditions were similar to those described in Table 1, with the following exceptions. In Expt. 1, the final concentration of MgSO_4 was 2 mM instead of 5 mM. In all three experiments only sodium ATP was used. In Expt. 3, the mitochondria were not washed and incubations were for 15 min. In Expt. 3b, 0.5 ml. of the mitochondrial suspension was added to 3.5 ml. of incubating medium in which the sodium and potassium phosphate buffers had been replaced by 10 mM-tris-HCl, pH 7.4. The final concentrations of ATP and L-glutamate in Expt. 3 were 1 and 20 mM respectively.

	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents (mitochondria/medium)	Sodium in mitochondria (m-equiv./l.)	Ratio of sodium contents (mitochondria/medium)
Expt. 1					
Whole kidney, control	22.6, 22.6	12.3, 12.5	1.50, 1.52	14.8, 14.8	1.81, 1.81
Whole kidney, ATP + glutamate	28.4 ± 0.1	21.0 ± 0.1	2.57 ± 0.02	19.9 ± 0.7	1.97 ± 0.07
Cortex, control	24.3 ± 0.1	13.0 ± 0.3	1.59 ± 0.03	15.0 ± 0.4	1.83 ± 0.04
Cortex, ATP + glutamate	26.3 ± 0.3	21.4 ± 0.3	2.61 ± 0.04	19.1 ± 0.3	1.89 ± 0.03
Medulla, control	18.3 ± 0.7	12.9 ± 0.4	1.57 ± 0.06	20.1 ± 0.7	2.45 ± 0.09
Medulla, ATP + glutamate	21.5 ± 0.2	14.1 ± 0.3	1.72 ± 0.04	23.7 ± 1.6	2.27 ± 0.15
Expt. 2					
Cortex, control	23.5 ± 0.4	14.3 ± 0.5	1.77 ± 0.07	21.8 ± 0.8	2.48 ± 0.10
Cortex, ATP + α -oxoglutarate	24.9 ± 0.4	27.7 ± 0.2	3.42 ± 0.03	25.1 ± 1.0	2.34 ± 0.09
Cortex, ATP + succinate	24.8 ± 0.3	28.1 ± 0.1	3.47 ± 0.01	25.7 ± 0.6	2.40 ± 0.07
Medulla, control	17.1 ± 0	13.4 ± 0.2	1.66 ± 0.03	25.9 ± 1.0	2.94 ± 0.12
Medulla, ATP + α -oxoglutarate	22.4, 20.2	18.8, 18.9	2.32, 2.34	33.0, 34.6	3.08, 3.23
Medulla, ATP + succinate	22.4, 21.8	19.1, 18.1	2.36, 2.24	34.7, 34.4	3.24, 3.21
Expt. 3					
Control (a)	19.4 ± 0.1	13.5 ± 0.3	1.18 ± 0.02	14.4 ± 0.1	1.13 ± 0.01
ATP + glutamate (a)	21.6, 22.1	22.8, 23.2	2.00, 2.04	17.2, 17.8	1.14, 1.18
Control (b)	19.1 ± 0.1	4.7 ± 0.1	3.62 ± 0.12	6.2 ± 0.5	4.77 ± 0.41
ATP + glutamate (b)	22.3 ± 0.1	14.2 ± 0.2	10.92 ± 0.17	8.9 ± 0.3	2.34 ± 0.09

Comparison of sodium and potassium transport by cortical and medullary mitochondria. Table 3 presents experiments in which movement of the two ions was studied with mitochondria isolated from the whole kidney, the cortex or the medulla. In the first experiment, with the 8–10 medium, the potassium gradient was maintained at a higher value by ATP and glutamate in mitochondria from the whole kidney and the cortex, than from medullary mitochondria. The sodium gradients did not change with the addition of ATP and substrate.

Since the medullary mitochondria maintained the potassium gradient with the addition of ATP and glutamate only slightly better than the control, this experiment was repeated with 5 mM- instead of 2 mM-MgSO₄. In this experiment a marked decrease in the loss of potassium occurred, the gradient being maintained at 3.06 in the presence of ATP and glutamate, where the control value was 1.66. The reason for these differences is not clear. It might be due to a higher magnesium requirement for potassium retention by the medullary mitochondria.

The second experiment (Table 3), which was also performed with the 8–10 medium, shows that with α -oxoglutarate or succinate the results obtained were similar to those with glutamate. However, the maintenance of the potassium gradients was significantly less with the medullary mitochondria than with those from the cortex. Measurement of the oxygen uptake by medullary mitochondria with the conventional Warburg technique with air as the gas phase and with the same media used for the test of potassium retention, showed that the uptake of oxygen with glutamate, α -oxoglutarate and succinate respectively was 237, 301 and 375.5 μ l. of air/2.5 ml. of suspension/ $\frac{1}{2}$ hr., the control value being 6.

When both the 0–1 medium and the 8–10 medium were used with the same preparation of unwashed medullary mitochondria (Table 3, Expt. 3), addition of ATP and glutamate to the medium resulted in significantly greater potassium gradients' being maintained by the particles. In Expt. 3b (Table 3), although the sodium content of the mitochondria incubated with ATP and glutamate was greater than that of the control, the sodium gradient was less. This is probably the result of using the sodium salt of ATP in the incubating medium, the resulting increase in the sodium concentration of the medium being greater than the increased sodium concentration in the mitochondria.

In comparing the movements of sodium, potassium and water by medullary and cortical mitochondria, a number of differences were found. First, the potassium gradients maintained by washed cortical mitochondria with ATP and sub-

strate usually were significantly greater than those maintained by the medullary mitochondria. Secondly, the water content of the medullary mitochondria was significantly greater than that of the cortical mitochondria. However, addition of ATP and substrate to the medium resulted in extrusion of water from both sorts of mitochondria. Thirdly, on a dry-weight basis, the amount of mitochondrial material isolated from the cortex was two to three times that from the medulla.

Experiments with the 0–1 medium. Table 4 shows that the loss of sodium from kidney mitochondria is different from that of potassium. In the first experiment, the mitochondria were suspended in a solution containing approx. 8 mM-sodium, and the suspension was added to a solution containing approximately the same amount of potassium but no sodium. ATP and α -oxoglutarate had virtually no effect on the sodium content, which decreased 26 % during 40 min. incubation. Loss of potassium was significantly decreased after 20 and 40 min. incubation by the addition of nucleotide and substrate.

In the second experiment, the mitochondrial suspension was added to a medium containing only the sodium and potassium contributed by the sodium and potassium salts of ATP. A greater potassium gradient was maintained when the nucleotide and α -oxoglutarate were added to the medium. Although the sodium content of the particles was somewhat greater with ATP and substrate, the sodium gradient was less than that of the control. This observation is similar to the one in Expt. 3b (Table 3).

The third experiment was designed primarily to determine whether a massive amount of ATP (25 mM) and substrate would cause an increase in the sodium gradient. It had been demonstrated that if arsenite was added to a suspension of heart mitochondria together with 1 mM-ATP and 20 mM- α -oxoglutarate, the usual increase in the potassium gradient was prevented (Ulrich, 1960a). This inhibition could be partially overcome by increasing the ATP concentration 25-fold. The third experiment shows that even with 25 mM-ATP, the sodium gradient after incubation did not differ from the incubated control. The potassium gradients, which reflect the loss of endogenous potassium from the mitochondria (no potassium having been present in either the suspending or incubating media), were greater with the addition of ATP and substrate, i.e. these two compounds caused decreased loss of the ion from the particles. In this third experiment, an amount of NaCl approximately equal to the sodium in the ATP was added to the control solutions.

Effects of pH. In these experiments, only the pH of the incubating media was varied, the pH of the

suspending medium, which in most of the experiments was 6.9–7.1, being kept constant. In the first experiment, with the 8–10 medium (Table 5), increasing the pH from 6.2 to 7.4 caused a significant decrease in the loss of potassium, the control potassium gradient increasing from 2.40 at pH 6.2 to 3.65 at pH 7.4. These changes did not occur when ATP and α -oxoglutarate were added to the incubating medium. In fact, the potassium gradients decreased slightly as the pH was increased. The loss of sodium from the mitochondria was virtually unchanged as the pH was altered.

The second experiment (8–10 medium) was designed to study changes in the loss of potassium over a wider pH range when the mitochondrial suspensions were centrifuged immediately after being added to the incubating media. The buffering range of the tris and phosphate buffers used is such that at the lowest pH values studied, the pH of the solutions increased from 4.5 to 5.6 and from 5.5 to

5.9 when 0.5 ml. of the suspending medium (not containing mitochondria) was added to 3.5 ml. of the incubating medium. Nevertheless, the results show that, as the pH of the incubating medium was raised, the loss of potassium was significantly decreased. Again there was virtually no change in the sodium content of the mitochondria.

The effects of pH on the potassium gradients are illustrated even more strikingly in the third experiment, in which the 0–1 medium was used. As the pH of the incubating medium was increased from 6.2 to 7.8, the control potassium gradient increased more than fourfold, from 4.92 to 20.70. Again, changes in pH had little, if any, effect on the potassium gradients when ATP and α -oxoglutarate were added to the incubating medium. The sodium gradient also was not affected.

That these effects of pH were not due to the pH of the incubating media, but probably to some sort of exchange of H^+ ion for K^+ ion, was shown by an experiment in which mitochondria were suspended

Table 4. *Effects of adenosine triphosphate and α -oxoglutarate on loss of sodium and potassium from rabbit-kidney mitochondria*

<p>In Expt. 1, the mitochondria were suspended with 0.25M-sucrose, 5 mM-MgSO₄ and 5 mM-NaH₂PO₄·H₂O-Na₂HPO₄ (pH 7.4). A portion (0.5 ml.) of the suspension was added to 3.5 ml. of incubating medium consisting of 0.25M-sucrose, 5 mM-MgSO₄ and 5 mM-K₂HPO₄-KH₂PO₄ (pH 7.4), together with (or without) potassium ATP and α-oxoglutarate to give a final concentration of 1 mM and 20 mM respectively. In Expt. 2, the mitochondria were suspended with the same solution as in Expt. 1. However, 5 mM-K₂HPO₄-KH₂PO₄ (pH 7.4) had been added to the solution. A portion (0.5 ml.) of the suspension was added to 3.5 ml. of incubating medium which consisted of 0.25M-sucrose, 5 mM-MgSO₄ and 10 mM-tris (pH 7.4) together with (or without) potassium ATP, sodium ATP and α-oxoglutarate to give a final concentration of 0.5 mM, 0.5 mM and 20 mM respectively. In Expt. 3, the mitochondria were suspended with 0.25M-sucrose, 5 mM-MgSO₄ and 10 mM-Na₂HPO₄-NaH₂PO₄·H₂O (pH 7.4). A portion (2 ml.) of the suspension was added to 2 ml. of incubating medium with the same composition together with (or without) sodium ATP and α-oxoglutarate to give a final concentration of 25 and 20 mM respectively. Incubation times are shown in parentheses after the details of the experiments.</p>					
	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents (mitochondria/medium)	Sodium in mitochondria (m-equiv./l.)	Ratio of sodium contents (mitochondria/medium)
Expt. 1					
Control (no incubation)	27.4, 26.9	26.7, 27.8	3.82, 3.92	9.6, 11.4	10.91, 12.96
ATP + α -oxoglutarate (no incubation)	27.5 $\pm$ 0.1	33.6 $\pm$ 0.5	3.86 $\pm$ 0.04	11.8 $\pm$ 0.7	11.89 $\pm$ 0.72
Control (20 min.)	25.6 $\pm$ 0.6	12.7 $\pm$ 0.1	1.72 $\pm$ 0.02	9.2 $\pm$ 0.6	10.54 $\pm$ 0.71
ATP + α -oxoglutarate (20 min.)	27.9 $\pm$ 0.1	28.3 $\pm$ 0.1	3.11 $\pm$ 0.02	9.4 $\pm$ 0.3	9.12 $\pm$ 0.30
Control (40 min.)	24.7 $\pm$ 0.6	11.8 $\pm$ 0.1	1.56 $\pm$ 0.01	8.0 $\pm$ 0.8	8.66 $\pm$ 0.86
ATP + α -oxoglutarate (40 min.)	27.4 $\pm$ 0.4	22.8 $\pm$ 0.3	2.48 $\pm$ 0.02	8.3 $\pm$ 0.5	8.17 $\pm$ 0.54
Expt. 2					
Control (no incubation)	25.6 $\pm$ 0.2	12.9 $\pm$ 1.0	11.73 $\pm$ 0.90	9.1 $\pm$ 0.1	11.37 $\pm$ 0.16
ATP + α -oxoglutarate (no incubation)	27.0 $\pm$ 0.3	20.1 $\pm$ 0	11.17 $\pm$ 0	10.4 $\pm$ 0.2	5.22 $\pm$ 0.09
Control (20 min.)	21.6 $\pm$ 0.1	3.7 $\pm$ 0.1	2.64 $\pm$ 0.04	6.3 $\pm$ 0.1	7.00 $\pm$ 0.08
ATP + α -oxoglutarate (20 min.)	24.9 $\pm$ 0.1	10.4 $\pm$ 0.2	4.95 $\pm$ 0.09	8.5 $\pm$ 0.3	4.25 $\pm$ 0.14
Control (40 min.)	19.8 $\pm$ 0	2.9 $\pm$ 0	2.15 $\pm$ 0	5.5 $\pm$ 0.1	6.11 $\pm$ 0.13
ATP + α -oxoglutarate (40 min.)	24.0 $\pm$ 0.1	6.1 $\pm$ 0.1	2.54 $\pm$ 0.02	7.7 $\pm$ 0.2	3.85 $\pm$ 0.11
Expt. 3					
Control (no incubation)	27.8 $\pm$ 1.03	26.6 $\pm$ 0.5	120.9 $\pm$ 2.5	84.1 $\pm$ 2.8	1.18 $\pm$ 0.04
ATP + α -oxoglutarate (no incubation)	27.5 $\pm$ 0.2	26.9 $\pm$ 0.3	149.3 $\pm$ 1.8	76.8 $\pm$ 3.0	1.10 $\pm$ 0.04
Control (20 min.)	27.0 $\pm$ 0.2	5.3 $\pm$ 0.1	8.03 $\pm$ 0.23	87.2 $\pm$ 1.8	1.23 $\pm$ 0.02
ATP + α -oxoglutarate (20 min.)	26.5 $\pm$ 0.5	14.0 $\pm$ 0.1	31.1 $\pm$ 0.2	86.9 $\pm$ 1.2	1.24 $\pm$ 0.02
Control (40 min.)	26.4, 26.6	4.7, 4.5	6.7, 6.3	88.2, 90.4	1.27, 1.24
ATP + α -oxoglutarate (40 min.)	28.6, 28.1	10.9, 10.4	19.3, 19.8	80.6, 82.1	1.15, 1.18

Table 5. *Effects of pH on sodium and potassium contents of rabbit-kidney mitochondria*

In Expt. 1 the mitochondria were suspended with the same solution as in Table 1. A portion (0.5 ml.) of the suspension was added to 3.5 ml. of incubating medium of the same composition together with (or without) sodium ATP and α -oxoglutarate to give final concentrations of 1 mM and 20 mM respectively. The pH of the incubating media was adjusted with either solid tris (ATP and α -oxoglutarate) or solid tris or HCl (control) or both. Suspensions were incubated for 20 min. In Expt. 2, no ATP or α -oxoglutarate was added and the suspensions were centrifuged immediately after addition of the mitochondria to the incubating medium. Figures in parentheses refer to the pH resulting when 0.5 ml. of the solution used for suspending the mitochondria was added to 3.5 ml. of the incubating medium. In Expt. 3, the mitochondria were suspended with the same solution as in Expts. 1 and 2. A portion (0.5 ml.) of the suspension was added to 3.5 ml. of a solution consisting of 0.25M-sucrose, 5 mM-MgSO₄ and 10 mM-tris-HCl buffer (pH 7.4). When added, sodium ATP, potassium ATP and α -oxoglutarate were present at final concentrations of 0.5, 0.5 and 20 mM respectively. Incubations were carried out for 15 min.

	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents (mitochondria/medium)	Sodium in mitochondria (m-equiv./l.)	Ratio of sodium contents (mitochondria/medium)
Expt. 1					
pH 6.2, control	26.6±0.3	19.9±0.1	2.40±0.01	16.7±0.2	2.04±0.03
pH 6.2, ATP + α -oxoglutarate	26.5±0.1	34.2±0.2	4.12±0.03	17.1±0.8	1.68±0.07
pH 6.8, control	26.7±0.1	26.6±0.3	3.20±0.03	18.2±1.0	2.22±0.12
pH 6.8, ATP + α -oxoglutarate	26.9±0.2	34.3±0.5	4.13±0.05	17.7±0.8	1.74±0.08
pH 7.4, control	28.5±0.1	30.3±0.3	3.65±0.03	18.3±0.4	2.23±0.05
pH 7.4, ATP + α -oxoglutarate	29.4±0.2	32.0±0.3	3.86±0.04	17.5±0.2	1.71±0.04
pH 7.8, control	28.0±0.1	30.4±0.2	3.66±0.02	17.3±0.2	2.11±0.03
pH 7.8, ATP + α -oxoglutarate	28.1±0.1	29.9±0.2	3.60±0.03	16.8±0.1	1.65±0.01
Expt. 2					
pH 4.5 (5.6)	28.4±0.1	20.6±0.2	2.58±0.03	18.9±0.8	2.42±0.10
pH 5.5 (5.9)	27.7±0.2	20.6±1.1	2.58±0.14	19.1±1.6	2.45±0.21
pH 6.4 (6.5)	26.6±0.4	21.9±0.3	2.74±0.03	17.6±0.7	2.26±0.09
pH 6.9 (6.9)	25.4±0.1	23.7±0.4	2.96±0.06	18.0±0.3	2.31±0.03
pH 7.4 (7.4)	26.5±0.5	28.8±0	3.60±0	19.7±0.2	2.53±0.02
pH 8.4 (8.3)	27.1±0.2	32.1±0.1	4.01±0.01	19.1±0.1	2.45±0.01
Expt. 3					
pH 6.2, control	26.5±0.9	6.4±0.3	4.92±0.23	12.8±0.6	14.22±0.65
pH 6.2, ATP + α -oxoglutarate	27.5±0.2	26.7±0.3	14.83±0.15	14.9±0.2	7.84±0.09
pH 6.8, control	26.5±0.1	7.1±0.3	5.46±0.23	14.5±0.9	16.11±0.96
pH 6.8, ATP + α -oxoglutarate	28.4±0	27.5±0.2	15.28±0.11	15.1±0.9	7.95±0.45
pH 7.4, control	28.6±0.3	11.8±0.3	9.83±0.22	12.1±0.7	13.45±0.76
pH 7.4, ATP + α -oxoglutarate	29.0±0.2	26.9±0.2	14.94±0.09	13.7±0.2	7.21±0.09
pH 7.8, control	29.2±0.3	20.7±0.5	20.70±0.47	12.6±0.5	14.00±0.59
pH 7.8, ATP + α -oxoglutarate	28.8, 28.4	24.4, 24.3	13.55, 13.50	13.3, 12.6	7.00, 6.64

in media at pH 6.2, 6.8 or 7.4 and added to incubating media with the same pH as the suspending media. Under these conditions, increasing the pH from 6.2 to 7.4 had no effect on the loss of potassium from the particles.

Effects of arsenite and 2:4-dinitrophenol on potassium gradients. It had been demonstrated (Ulrich, 1960a) that a variety of metabolic poisons, including those which uncouple oxidative phosphorylation, inhibited an increase in the potassium gradient maintained by rabbit-heart mitochondria. In the present study with the 0.1 medium containing AMP and α -oxoglutarate, addition of relatively large concentrations of arsenite and 2:4-dinitrophenol caused an increased loss of potassium from the mitochondria (Table 6, Expt. 1). In the second experiment, carried out with twice the concentration of potassium and no magnesium in

the medium, the effects of 2:4-dinitrophenol were more striking. At concentrations of 1 or 0.1 mM, the loss of potassium was greater than in the first experiment. The effect of 1 mM-2:4-dinitrophenol was similar whether AMP or ATP was used.

Other experiments designed to demonstrate an increase in the sodium gradient. A number of other experiments were performed in an attempt to demonstrate increased sodium gradients in the presence of ATP and substrate. These experiments were carried out with the 8-10 medium. Thus 2 mM-pyridoxal, alone or with ATP, was without effect. Incubating the mitochondria in the usual media but with 0.125M-, 0.25M- or 0.50M-sucrose also had no effect. Finally, an experiment was performed to rule out the possible effect of changes in ion gradients during the centrifuging that follows incubation of the mitochondria. The suspensions

Table 6. *Effects of 2:4-dinitrophenol and arsenite on loss of potassium from rabbit-kidney mitochondria*

In Expt. 1 the mitochondria were suspended with the same solution as in Table 1. A portion (0.5 ml.) of the mitochondrial suspension was added to 3.5 ml. of 0.25M-sucrose, 5 mM-MgSO₄ and 10 mM-tris-HCl (pH 7.4) together with sodium AMP and α -oxoglutarate to give a final concentration of 1 mM and 20 mM respectively. In Expt. 2 the mitochondria were suspended with 0.25M-sucrose and 10 mM-KH₂PO₄-K₂HPO₄ (pH 7.4), and 0.5 ml. of the suspension was added to 3.5 ml. of 0.25M-sucrose and 10 mM-tris-HCl (pH 7.4). The final concentration of sodium ATP was 1 mM. In both experiments, the mitochondria were incubated for 15 min. DNP, 2:4 Dinitrophenol.

	Percentage dry wt.	Potassium in mitochondria (m-equiv./l.)	Ratio of potassium contents (mitochondria medium)
Expt. 1			
Control	23.8 $\pm$ 0.3	7.5 $\pm$ 0.1	6.25 $\pm$ 0.05
AMP + α -oxoglutarate	26.2 $\pm$ 0.3	21.4 $\pm$ 0.3	17.84 $\pm$ 0.21
AMP + α -oxoglutarate + 1 mM-arsenite	25.3 $\pm$ 0.2	7.2 $\pm$ 0.3	6.00 $\pm$ 0.27
AMP + α -oxoglutarate + 1 mM-DNP	26.9, 28.5	16.0, 17.3	13.33, 14.42
AMP + α -oxoglutarate + 0.01 mM-arsenite	29.0 $\pm$ 0.3	20.4 $\pm$ 0.3	17.00 $\pm$ 0.27
AMP + α -oxoglutarate + 0.01 mM-DNP	28.9 $\pm$ 0.1	18.8 $\pm$ 0.3	15.66 $\pm$ 0.28
Expt. 2			
Control	21.6 $\pm$ 0	6.2 $\pm$ 0.1	2.14 $\pm$ 0.03
AMP + α -oxoglutarate	26.9 $\pm$ 0	21.4 $\pm$ 0.6	7.38 $\pm$ 0.19
AMP + α -oxoglutarate + 1 mM-DNP	26.4 $\pm$ 0.3	9.3 $\pm$ 0.3	3.21 $\pm$ 0.10
AMP + α -oxoglutarate + 0.1 mM-DNP	25.2 $\pm$ 0.6	10.0 $\pm$ 0.1	3.45 $\pm$ 0.03
AMP + α -oxoglutarate + 0.01 mM-DNP	26.8, 26.8	16.9, 16.6	5.83, 5.72
ATP + α -oxoglutarate + 1 mM-DNP	25.8 $\pm$ 0.3	9.9 $\pm$ 0.2	3.41 $\pm$ 0.05

were centrifuged for either the standard 5 min. or for 1 or 2 min. After the 5 min. centrifuging, half the tubes were allowed to stand for 10 min. before the supernatant was poured off. The only significant change in this experiment was that the percentage dry weight of the mitochondria isolated after 1 or 2 min. centrifuging (25.6 %) was less than that of the 5 min. centrifuging (27.9 %). ATP and α -oxoglutarate did not increase the sodium gradient. A slight decrease of the sodium gradient occurred with these additions (from 1.87 to 1.55), but these changes were so small that their significance is doubtful.

DISCUSSION

Possible reasons for inability to demonstrate increased sodium gradients. One of the main objects of the present study was to determine whether the sodium gradients developed by kidney mitochondria during incubation at 37° could be increased in the presence of an oxidizable substrate and ATP. In other words, whether metabolic activity could decrease the loss of sodium from the particles. Since the evidence presented in this paper is completely negative on this point, it is necessary to consider whether this might be due to the conditions of the experiments.

The sodium and potassium concentrations in the mitochondria were calculated from the water content of the whole mitochondrial pellet. This would include intermitochondrial (extraparticulate) water which Werkheiser & Bartley (1957) and Jackson & Pace (1956) estimated to account for 20 % or less of

the total water of liver-mitochondrial pellets. Amoore & Bartley (1958) reported variations as great as 24–59 % in the extraparticulate water of liver mitochondria from different preparations. It is possible therefore that changes in intramitochondrial sodium could be obscured by extraparticulate sodium, especially if the extraparticulate water content were as great as 59 %. Although the response was not uniform, in most of the experiments addition of ATP and substrate to the mitochondrial suspension resulted in extrusion of water from the particles. If sodium were not extruded with water, its concentration in mitochondrial water could have risen without a marked change in the sodium content of the pellet. However, it is doubtful whether the small changes in mitochondrial water would have obscured marked changes in mitochondrial sodium.

Although these two criticisms cannot be answered satisfactorily, the decreased loss of potassium in the presence of ATP and substrate was of such magnitude that small variations in the extraparticulate concentration of this ion could not have caused the observed changes. Since these experiments were designed to measure only changes in relative ion concentration, the contribution of the extraparticulate water can probably be disregarded if one assumes that it is constant in each mitochondrial preparation (cf. Jackson & Pace, 1956).

The inability to demonstrate increased sodium gradients might have been due to loss of these gradients between the time that incubation was

stopped and the supernatant removed after centrifuging of the suspension. Also, since the mitochondria were not washed after incubation and centrifuging, an increased sodium gradient might have been obscured if the increase were limited to a particular sodium fraction.

Mitochondrial sodium studies by other investigators. A number of investigators have reported that the sodium and potassium gradients developed by sheep-kidney mitochondria (Bartley & Davies, 1954) and rat-liver mitochondria (Macfarlane & Spencer, 1953; Price, Fonnesu & Davies, 1956) could be increased by incubation in the presence of an oxidizable substrate and an adenine nucleotide. The experimental conditions of the present study, although not identical, were similar to those of these three groups. Their data for sodium was so variable that the results must be viewed with caution. With kidney mitochondria, Bartley & Davies (1954) usually were unable to demonstrate concentration gradients greater than 1.0 for sodium or potassium when they studied the distribution of the radioactive ions between the particles and medium. In the same paper (Table 7), Bartley & Davies presented data not only for the development of both sodium and potassium gradients but also for significant increases in these gradients in the presence of α -oxoglutarate and ATP.

Macfarlane & Spencer (1953) observed that the sodium and potassium contents of mitochondria incubated for 10 min. in a medium containing AMP and substrate were low, but doubled in the next 20 min. incubation. This could not be confirmed by Price *et al.* (1956), who carried out a detailed study of ion and water movements by rat-liver mitochondria. Although Price and co-workers were able to demonstrate consistently increased potassium gradients when ATP or AMP was added to a medium containing oxidizable substrate, their results for increased sodium gradients were not consistent. Price and co-workers also studied the effects of varying the concentrations of phosphate buffer and Mg^{2+} ion (their Table 6). They found that increasing the phosphate concentration 10- or 30-fold had no effect on sodium transport. However, decreasing the Mg^{2+} ion concentration from 20 to 2 mM resulted in increased sodium gradients when AMP was added to the medium. In the present series of experiments, decreasing the magnesium concentration from 5 to 2 mM had no effect on sodium transport (compare Expts. 1 and 2 in Table 1 with Expt. 1 in Table 3).

Perfusion of the kidneys *in situ* with iso-osmotic salt solution and sucrose caused the disappearance of a small amount of dark-red pigment at the bottom of the mitochondrial pellet, but had no effect on the loss of sodium and potassium when

compared with isolated mitochondria from unperfused kidneys.

It is possible that the differences between the results in this paper and those of the three groups mentioned above are due to species differences. However, both Spector (1953) and Jackson & Pace (1956) found that the sodium gradients maintained by rat-liver mitochondria could not be increased.

Potassium transport. The greater potassium gradients maintained by rabbit-kidney mitochondria in the presence of an adenine nucleotide and oxidizable substrate confirms previous observations on mitochondria from sheep kidney (Bartley & Davies, 1954), rat liver (Macfarlane & Spencer, 1953; Spector, 1953; Price *et al.* 1956), rat heart and skeletal muscle (Ulrich, 1959) and rabbit heart (Ulrich, 1960a). Does this mean that potassium is actively transported by mitochondria? In a study with isolated heart mitochondria in which a variety of metabolic inhibitors were added to the incubating media, some evidence was presented for possible active transport (Ulrich, 1960a). However, lack of knowledge of the potential difference across the mitochondrial membrane makes it difficult to prove this point conclusively. A discussion of this question is given by Amore (1960). He found a lower concentration of chloride within the mitochondria than in the medium, which suggested that there might be a potential difference of the correct sign.

One series of observations in this study which is difficult to explain are the effects of pH on the potassium gradients. The increased gradients resulting from increasing the pH of the incubating medium might be due to a competition of H^+ and K^+ ions for a common site on a membrane complex or carrier. Since the gradients appeared to have reached a maximum when ATP and substrate were added to the incubating medium, it is not surprising that under these conditions changes in pH appeared to have no effect.

The sodium gradients maintained by mitochondria were not affected by changes in pH. Two observations may have a bearing on this finding. First, when heart or skeletal-muscle mitochondria are aged, the potassium content decreases approximately by one-half whereas the sodium content remains the same (Ulrich, 1959). Secondly, the specific activity of sodium ($\mu g.$ of sodium/mg. dry wt.) was found to be several times as great in mitochondrial membrane fractions as in intact particles, whereas the reverse was found with potassium (Ulrich, 1960b). One interpretation of the lack of effect of pH is that mitochondrial sodium is located in the membrane, possibly firmly bound, and cannot compete with H^+ ions for some common site on the membrane.

SUMMARY

1. The loss of sodium and potassium from isolated rabbit-kidney mitochondria was studied. Addition of ATP and an oxidizable substrate to the incubating medium at 37° caused a significant decrease in the loss of potassium from the mitochondria, but had no effect on the sodium content of the particles.

2. Increasing the pH of incubating medium not containing adenosine triphosphate and substrate by small increments from 6.2 to 7.8 resulted in a significant decrease in the loss of potassium from the mitochondria. These changes in potassium were not observed when adenosine triphosphate and substrate were added to the medium. The sodium content of the particles was not altered by changes in pH.

3. Mitochondria isolated from either the cortex or the medulla of the kidney treated sodium and potassium in a manner similar to those from the whole kidney. However, adenosine triphosphate and substrate did not appear to inhibit loss of potassium from medullary mitochondria as effectively as from cortical mitochondria.

4. Addition of arsenite or 2:4-dinitrophenol to a medium containing adenosine monophosphate and substrate caused a marked increase in the loss of potassium.

5. Possible explanations are presented for the inability to demonstrate decreased loss of sodium

in the presence of an oxidizable substrate and adenine nucleotide, and some conflicting results of sodium movements in liver and kidney mitochondria by other investigators are discussed.

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REFERENCES

- Amoore, J. E. (1960). *Biochem. J.* **76**, 438.
 Amoore, J. E. & Bartley, W. (1958). *Biochem. J.* **69**, 223.
 Bartley, W. & Davies, R. E. (1954). *Biochem. J.* **57**, 37.
 Jackson, K. L. & Pace, N. (1956). *J. gen. Physiol.* **40**, 47.
 Macfarlane, M. G. & Spencer, A. G. (1953). *Biochem. J.* **54**, 569.
 Pauly, H., Packer, L. & Schwan, H. P. (1960). *J. biophys. biochem. Cytol.* **7**, 589.
 Price, C. A., Fonnesu, A. & Davies, R. E. (1956). *Biochem. J.* **64**, 754.
 Spector, W. G. (1953). *Proc. Roy. Soc. B*, **141**, 268.
 Ulrich, F. (1959). *Amer. J. Physiol.* **197**, 997.
 Ulrich, F. (1960a). *Amer. J. Physiol.* **198**, 847.
 Ulrich, F. (1960b). *Nature, Lond.*, **187**, 949.
 Ulrich, F. (1961). *Fed. Proc.* **20**, 145.
 Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.

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Acetoacetate as Fuel of Respiration in the Perfused Rat Heart

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Kulka, Krebs & Eggleston (1961) found that sheep-heart homogenates readily oxidize added acetoacetate. The ratio (oxygen used/acetoacetate oxidized) reached values of up to 4, indicating that acetoacetate was the sole or main fuel of respiration. It suppressed the oxidation of endogenous substrates.

Experiments on the isolated perfused rat heart are reported in this paper which show that the intact heart of the rat likewise uses acetoacetate as a preferred substrate of oxidation, even in the presence of glucose and insulin.

EXPERIMENTAL

Perfusion method. The perfusion apparatus was similar to that described by Bleehen & Fisher (1954) except that the anti-frothing device was omitted as frothing presented no difficulty. The gas mixture of O₂ + CO₂ (95:5, v/v) was saturated with water vapour at 37° before being passed to the gas lifts, in order to avoid loss of water from the perfusion fluid by evaporation. The perfusate volume was usually between 17 and 25 ml. The bath temperature was 37°.

Male rats (Wistar strain) weighing between 220 and 280 g. were used. 'Well-fed' animals had unrestricted