# Effects of Sodium and Potassium Ions on Oxidative Phosphorylation in Relation to Respiratory Control by a Cell-Membrane Adenosine Triphosphatase

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1. A study has been made of the oxygen consumption of kidney homogenates in relation to the ADP concentration as regulated by the cell-membrane adenosine triphosphatase. Stimulation of this enzymic activity by $Na^+$ and $K^+$ caused parallel increases in oxygen consumption and ADP concentration. Similarly, inhibition with ouabain caused a parallel fall. The membrane adenosine triphosphatase concerned in active transport therefore appears to regulate respiration through its control of ADP concentration. 2. The respiration of homogenates and mitochondria was also stimulated by $K^+$ in a way independent of adenosinetriphosphatase activity. It was shown that $K^+$ facilitates oxidative phosphorylation and the respiratory response to ADP. A $K^+$ concentration of 25–50mM was needed for maximum oxidative phosphorylation in the presence of physiological concentration of $Na^+$. $Na^+$ counteracted $K^+$ in the effects on mitochondria. It is concluded that $K^+$ regulates cellular respiration at two structures, one directly in mitochondria, and the second indirectly through control of ADP production at the cell membrane.

The regulation of respiration has been investigated mainly with mitochondria and has given rise to the valuable concept that one factor involved is the ambient ADP concentration. It is now widely believed that ADP, as phosphate acceptor for oxidative phosphorylation, can control the rate of respiration in a tightly coupled system. The evidence is particularly strong, largely as a result of varying the ADP concentration directly or doing this indirectly by changing the ATPase† activity (Lardy & Wellman, 1952; Chance & Williams, 1956). Because of analytical difficulties, however, very few tests of this concept have been made with intact cells or even homogenates, yet it would be expected that their respiration is also regulated in the same way. Thus cells that are engaged in physiological activity have higher rates of respiration than cells at rest, and the regulation is reasonably attributed to the ATPase method of control since ATP is utilized for work processes. ADP produced in other cell structures would thus be able to control mitochondrial respiration.

Active transport at the cell membrane is one process that utilizes ATP, and in many tissues the initiation of osmotic work is accompanied by a raised oxygen consumption or lactate production. Energy production is geared to energy utilization. In kidney-cortex slices, for example, there is a close parallelism between oxygen consumption and $K^+$ uptake (Whittam, 1961; Burg & Orloff, 1962; Whittam & Willis, 1963). In attempting to elucidate the mechanism of this interdependence, it was found in kidney homogenates that the same parallelism also obtained between oxygen consumption and the activity of the cell-membrane ATPase (Blond & Whittam, 1964*a*). The question therefore arises whether the ADP concentration in the homogenate rises with an increase in the rates of ATP hydrolysis and of oxygen uptake.

Another aspect of respiratory control by ions is the possibility of direct effects on mitochondria, for, besides the indirect effect of $K^+$ on respiration via ATPase activity, it became evident that $K^+$, in addition, had a direct activating effect on mitochondria that was not due to an increase in ATPase activity either of mitochondria or other cell components. A stimulation of respiration by $K^+$ in liver mitochondria was described by Lardy (1951, 1955) and Pressman & Lardy (1955). $K^+$ caused an increased response to ADP and had no effect in the absence of phosphate acceptors. The results in the present paper show, first, that the respiration of homogenates is linearly related to the ADP concentration when the latter is varied by changing the cell-membrane ATPase activity. Secondly, the action of $K^+$ on kidney mitochondria is shown to

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† Abbreviation: ATPase, adenosine triphosphatase.

cause stimulation of oxygen consumption and to increase the efficiency of oxidative phosphorylation. A preliminary account of this work has been made (Blond & Whittam, 1964*b*).

## METHODS

Adult rabbits of both sexes were stunned by a blow on the neck and bled from the throat. The kidneys were removed as soon as possible after death. After removal of the capsule, each kidney was bisected longitudinally and the exposed medulla cut away. The cortex (about 4g.) was disrupted in about 12ml. of ice-cold medium containing sucrose (0·25M) and imidazole–HCl buffer, pH7·8 (10mM), in a Potter–Elvehjem-type homogenizer with a smooth glass tube and Teflon pestle (total clearance 0·01in.). The pestle, revolving at 700rev./min., was taken 20 times up and down through the suspension, which was then squeezed through a double layer of muslin and the volume adjusted to give a 20% (w/v, referred to wet wt. of tissue) suspension by the addition of more 0·25M-sucrose medium.

*Preparation of kidney mitochondria.* A 10% (w/v) homogenate was centrifuged at 850*g* for 10min. at 0° (MSE Refrigerator centrifuge, 6886 head, 2300–2400rev./min.) to spin down the cellular debris and nuclei. This procedure was repeated on the supernatant liquid, and the two sediments were discarded. The mitochondria were then sedimented at 5000*g* for 15min., and after being washed once in 0·25M-sucrose medium they were resuspended, and diluted with sucrose medium to give a final volume of about 15ml. On the basis of dry weight, the mitochondrial fraction constituted 15% of the total dry weight of the homogenate.

*Measurement of respiration.* The rates of $O_2$ consumption were measured by incubation at 37° in air, by using the conventional Warburg manometer. Portions (1·0ml.) of 20% (w/v) homogenate (30–40mg. dry wt.) or samples of a suspension of mitochondria (usually about 10mg. dry wt./ml.) were added to manometer cups containing 3·0ml. of medium of composition (final concentrations): imidazole–HCl buffer, pH7·8 (15mM), tris–malate buffer, pH7·8 (10mM), $MgCl_2$ (2mM), $NAD^+$ (0·125mM), ATP (disodium salt) (1·25mM), with NaCl and KCl concentrations as specified in the text. It was necessary to add both ATP and $NAD^+$ to the medium to obtain linear rates of respiration. $NAD^+$ was added to compensate for the destruction in homogenates of this cofactor, which is essential for the malate-dehydrogenase system. Incubation of homogenates was commenced 0·5–1hr. after removal of the kidney from the animal, and that of mitochondria after 2·5–3hr. Oxidative activity ($Q_{O_2}$) is expressed as $\mu$l. of $O_2$ uptake/mg. dry wt./hr.

*Assay of adenine nucleotides.* Analyses were made on tissue homogenates after measurement of their rates of respiration in Warburg manometers. A 0·1ml. sample of 70% (v/v) $HClO_4$ was added to the main compartment of the flask from the side arm, and, as rapidly as possible, the contents of the flask were poured into a tube standing in ice–water. The precipitated protein was spun down and a sample (2·0ml.) of supernatant pipetted into a weighed tube. The solution was neutralized (pH6·5–7·5) with 2N-KOH by using B.D.H. universal indicator (British Drug Houses Ltd., Poole, Dorset) externally. The tube was then reweighed to obtain the total volume of liquid, and placed in ice for 20–30min. to allow complete precipitation of the $KClO_4$. Samples (0·1–0·5ml.) of the clear extract were used for the estimation of ATP, ADP and AMP.

(*a*) Assay of ATP. A Boehringer Test Kit for ATP was used and modified for a total of 1ml. in the cuvettes. When different amounts of ATP were added to homogenate containing $HClO_4$, and the ATP was subsequently estimated, 95% of the added ATP was measured in the assay procedure. ADP or AMP at concentrations of 2mM did not interfere with the measurement of ATP.

(*b*) Assay of ADP and AMP. The Boehringer Test Kit for the assay of ADP and AMP in blood was modified for the use of 1·0ml. final volume of reactants in the cuvette, and replacement of the triethanolamine–$K_2CO_3$ buffer with triethanolamine alone. This modification was more convenient as the tissue extract was neutralized before the assay. The recovery of different amounts of ADP or AMP added to homogenates denatured with $HClO_4$ was 92–102%. No correction was made for traces of AMP that may have been present in the Boehringer Test Kit (but see Chappell, 1964). The presence of adenine nucleotides (2mM), other than that being measured, did not interfere with the estimation of 0·2mM-nucleotide.

Reproducibility of these methods was estimated by incubating, under identical conditions, six flasks containing kidney homogenates respiring in medium containing KCl (100mM) and NaCl (50mM). The contents of each flask were analysed for ATP, ADP and AMP, and the results (expressed as the concentrations in the incubated suspension) were: ATP, 0·380mM (±4% S.E.M.); ADP, 0·345mM (±4·5% S.E.M.); AMP 0·340mM (±3% S.E.M.).

Other nucleotides react quantitatively in the assay system used here for the estimation of adenine nucleotides (Kornberg, 1950), but they can be safely ignored on account of their low concentration and the relatively enormous amounts of adenine nucleotides in the reaction mixture.

*Measurement of phosphorylation quotient.* Kidney mitochondria were incubated in Warburg manometer flasks containing medium supplemented with glucose (20mM) and tris–phosphate buffer, pH7·8 (20mM). NaCl, KCl or ouabain were present as specified. After a 10min. thermal equilibration period, 30 i.u. of hexokinase (Sigma, type III) in 1% (w/v) glucose solution was tipped from the side arm into the main compartment. The $O_2$ uptake was measured during the subsequent 20–30min., when 100–200$\mu$l. of $O_2$ was consumed. The incubation was terminated by removal of manometers from the bath and, as quickly as possible, pouring the contents of the flasks into tubes containing 0·1ml. of 70% $HClO_4$. The precipitated protein was spun down and $P_i$ in the supernatant measured by the method of Fiske & Subbarow (1925). The amount of $P_i$ esterified during the period of measurement of $O_2$ consumption was obtained by subtraction of the final $P_i$ content of the incubation mixture from that present at the time of addition of hexokinase. The latter was estimated by incubating all flasks in duplicate, and, when hexokinase was tipped into one after 10min. equilibration, the reaction in the other was terminated by the addition of $HClO_4$. The $P_i$ in this flask was then estimated as above.

The accuracy of measurement of the P/O ratio in a single experiment was estimated by measuring the ratio for tissue incubated in six flasks under identical conditions. In a medium containing $K^+$ (100mM) and $Na^+$ (50mM), the mean P/O ratio was 1·85±0·11 (S.E.M.).

*Measurement of tissue concentration.* This was carried out as described by Blond & Whittam (1964*a*).

## MATERIALS

Imidazole, nicotinamide, choline chloride, phosphoric acid, trichloroacetic acid and ouabain were laboratory reagent products of British Drug Houses Ltd. The disodium salts of ADP and ATP were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., as was the tris (Sigma 7–9) and the yeast hexokinase (type III). L-Malic acid was a product of L. Light and Co. Ltd., Colnbrook, Bucks., and bovine serum albumin (fraction V) was obtained from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. Pyruvate kinase, lactate dehydrogenase, adenylate kinase, hexokinase, phosphoenolpyruvate (tricyclohexylammonium salt), triethanolamine hydrochloride, $NAD^+$, NADH, and the ATP and ADP–AMP Test Kits were all obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. All other compounds used were AnalaR reagents (British Drug Houses Ltd.).

All solutions were prepared in glass-distilled water. pH measurements were made with a glass electrode (model 23A; Electronic Instruments Ltd., Richmond, Surrey). Solutions of ATP and ADP were buffered to the required pH with saturated tris solution and kept at 0–4°.

## RESULTS

### *Nucleotides and the respiration of homogenates*

*Adenosine diphosphate.* Respiration of homogenates was varied by inducing changes in ATPase activity by interchanging $Na^+$ and $K^+$ concentrations. ADP was measured and was found to vary directly with the rate of respiration (Fig. 1). Thus, on raising the $Na^+$ concentration in the medium from 0 to 50mM, there was the same proportional increase in both the rate of respiration and the ADP concentration. These changes did not occur when ouabain was added, showing that they arise from an increase in activity of the cell-membrane ATPase. The mitochondrial ATPase activity is unaffected by ouabain and by the $Na^+$ and $K^+$ concentrations. The rise in respiration on adding $Na^+$ was from 24 to 33 $\mu$l./mg. dry wt./hr., and can therefore be ascribed to the rise in ADP concentration (from 0·3 to 0·4mM) resulting from the stimulation (by $Na^+$ and $K^+$) of the ouabain-sensitive ATPase. In the presence of ouabain, in contrast, the rate of respiration (24 $\mu$l./mg. dry wt./hr.) and the ADP concentration (0·3mM) remained unaffected by changes in $Na^+$ concentration from 0 to 50mM and in $K^+$ concentration from 150 to 100mM.

The close correlation that held on changing $Na^+$ and $K^+$ concentrations (with more than 50mM-$K^+$) was also found with different concentrations of ouabain. Fig. 2 shows that partial inhibitions of respiration with ouabain caused parallel changes in ADP concentration. The two continuous lines in Fig. 2 represent the effects on ADP concentration and the rate of respiration of changes in the membrane ATPase activity (shown by the broken line) that is activated by $Na^+$ and inhibited by ouabain. The ATPase was either activated by raising the

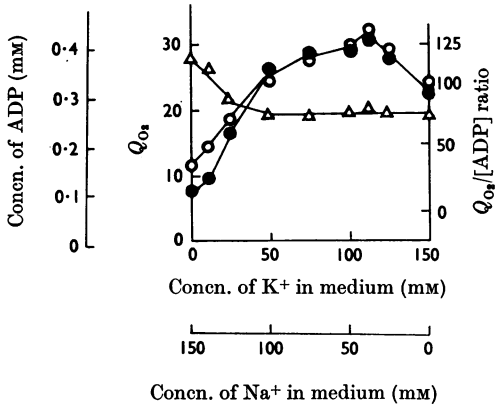


Fig. 1. Respiration and ADP concentrations in kidney-cortex homogenate. The $O_2$ uptake was measured manometrically in a standard medium containing L-malate (tris salt) (10mM), imidazole–HCl buffer, pH 7·8 (15mM), $MgCl_2$ (2mM), ATP (disodium salt) (1·25mM), $NAD^+$ (0·125mM) and the amounts of NaCl and KCl indicated. Manometer flask contents were analysed enzymically for ADP (Boehringer Test Kit). ○, $Q_{O_2}$ ($\mu$l. of $O_2$/mg. dry wt./hr.); ●, ADP concn.; △, $Q_{O_2}$/[ADP] ratio. Points are means taken from two to five experiments.

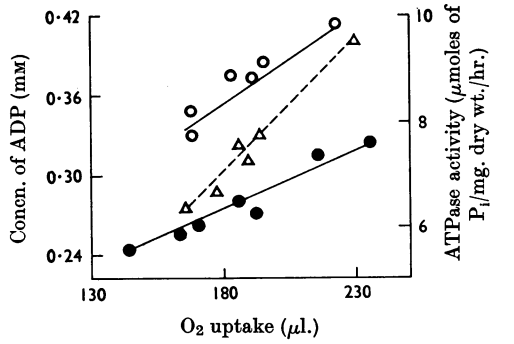


Fig. 2. Dependence of $O_2$ uptake on ADP concentration controlled by $Na^+$-plus-$K^+$-activated ATPase. ○, With variations in $K^+$ concn. from 50 to 150mM and in $Na^+$ concn. from 0 to 100mM; ●, graded inhibition by submaximal concentrations of ouabain, at 100mM-$K^+$ and 50mM-$Na^+$. Four experiments were performed, each with similar results; values taken from a single experiment are given. The broken line (△) shows the dependence of respiration on the $Na^+$-plus-$K^+$-activated ATPase activity, measured according to the method of Blond & Whittam (1964*a*), when this also is partially inhibited by ouabain.

$Na^+$ concentration in the presence of at least 50mM-$K^+$, or it was inhibited by ouabain in the presence of 100mM-$K^+$ and 50mM-$Na^+$. The changes in respiration resulting from changes in ATPase activity therefore appear to be mediated by alterations in the ADP concentration.

The parallelism between the rate of respiration and ADP concentration held for $K^+$ concentrations between 50 and 150mM, but below 50mM there was a bigger fall in ADP concentration than in the rate of respiration. This disparity is shown clearly in a plot of the ratio of the rate of respiration to ADP concentration (Fig. 1). The value of the ratio is constant with more than 50mM-$K^+$ but rises gradually by about 60% as the $K^+$ concentration in the medium is lowered to zero. This finding raises the question whether oxidative phosphorylation is depressed with a $K^+$ concentration of less than 50mM.

*Respiration of mitochondria*

At $K^+$ concentrations less than 50mM, the respiration of the homogenate was determined by the $K^+$ concentration in a way that was neither dependent on the presence of $Na^+$ nor inhibited by ouabain. This behaviour was also found in the respiration of isolated mitochondria, and cannot therefore be attributed to the presence of the $Na^+$-plus-$K^+$-activated ATPase.

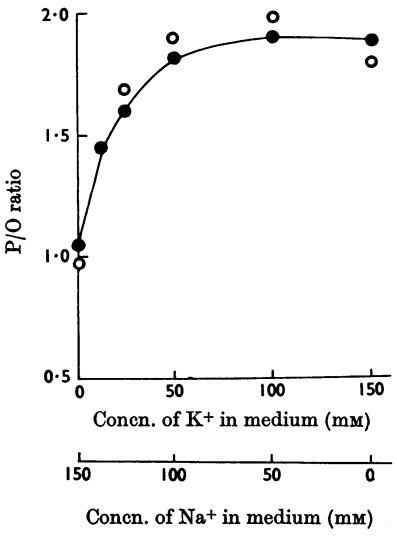


Fig. 3. Effect of $Na^+$ and $K^+$ on the P/O ratio of kidney-cortex mitochondria. Mitochondria were incubated in medium containing tris–malate buffer (10mM), glucose (20mM) and tris–phosphate buffer, pH 7·8 (20mM); 30 i.u. of hexokinase were added after thermal equilibration. The $O_2$ uptake was measured during the subsequent 20–30min.; the amount of $P_i$ esterified during this period was taken as the difference between the amount present after equilibration and that remaining at the end of incubation. ●, Control; ○, with ouabain (0·25mM). Points are means taken from four to six experiments.

Measurements of the P/O ratio were made at various $Na^+$ and $K^+$ concentrations to clarify the mechanism of the influence of $K^+$ on mitochondrial respiration. The results (Fig. 3) show that the P/O ratio increased from about 1·0 in a $K^+$-free medium to 1·9 when more than 50mM-$K^+$ and less than 100mM-$Na^+$ were present. The maximum experimental value of 1·9 for the P/O ratio is considerably lower than the theoretical value of 3; however, low P/O ratios are generally associated with oxidation of malate (Potter, 1945, 1947). The relatively high pH (7·8) used in this work to obtain maximum $Na^+$-plus-$K^+$-stimulated ATPase activity may also be a contributory factor to low P/O ratios, since Cooper & Lehninger (1956) have shown that the P/O ratio in liver mitochondria is maximum at pH 6, and decreases to about one-third of this value at pH 8. Moreover, as fluoride was not added in our experiments, the ATPase activity would be high and this would cause a low P/O ratio.

Simple substitution of $K^+$ for $Na^+$, as in Fig. 3, fails to establish whether the resultant effects are due to removal of one cation or addition of the other. To distinguish whether the changes in $Q_{O_2}$ and P/O ratio were due to stimulation by $K^+$ or to release of inhibition by $Na^+$, the latter was replaced by osmolar equivalents of sucrose, choline chloride or tris. The results in Table 1 show that, when $K^+$ (25mM) was added to the medium, the P/O ratio increased whether $Na^+$ was present or not. The effect was seen with each replacement for $Na^+$, indicating a specific effect of $K^+$. These substituents did not, however, cause the same decrease in P/O ratio on removal of $K^+$ from the medium; with choline and tris, the P/O ratio in a $K^+$-free medium was 0·7–0·8, as opposed to 0·4–0·5 in a medium containing $Na^+$ or sucrose. This may mean that choline and tris are themselves able to increase the tightness of coupling, or alternatively that sucrose or $Na^+$ decreases the P/O ratio. Since $K^+$ is firmly bound by mitochondria (Gamble, 1957; Rasmussen, 1964), these ions might be responsible for maintaining the remaining oxidative phosphorylation in $K^+$-free media. If this is the case, then the different effects of $Na^+$, sucrose, choline and tris on the P/O ratio might be related to their capacity for displacing or competing with $K^+$ for sites in the mitochondria.

Despite the uncertainty in interpreting fully the data in Table 1, the results nevertheless show that the increase in $Q_{O_2}$ and P/O ratio is due to stimulation caused specifically by $K^+$.

The use of foreign replacement compounds or ions is not altogether a satisfactory procedure.

Table 1. *Effect on the* P/O *ratio and respiration of kidney mitochondria of replacement of* $Na^+$ *in the medium with sucrose, chlorine or tris*

Incubation and analytical procedures were as described in Fig. 3. Tris was adjusted to pH 7·8 with 5 N-HCl before use.

| Concn. in medium (mM) | | $O_2$ uptake ($\mu$g.atoms of O/hr.) | P/O ratio | Percentage increase on addition of $K^+$ (25 mM) | |
|---|---|---|---|---|---|
| NaCl | KCl | | | $O_2$ uptake | P/O ratio |
| 150 | 0 | 25·5 | 0·4 | | |
| 125 | 25 | 55·5 | 1·3 | 120 | 225 |
| Sucrose | | | | | |
| 300 | 0 | 34·5 | 0·5 | | |
| 250 | 25 | 61·5 | 1·5 | 80 | 200 |
| Choline chloride | | | | | |
| 150 | 0 | 48·0 | 0·8 | | |
| 125 | 25 | 57·0 | 1·0 | 20 | 25 |
| Tris | | | | | |
| 150 | 0 | 30·0 | 0·7 | | |
| 125 | 25 | 72·0 | 1·2 | 140 | 70 |

Table 2. *Dependence of the* P/O *ratio of kidney-cortex mitochondria on the relative concentrations of* $Na^+$ *and* $K^+$

Experimental details were as described in Fig. 3.

| | Concn. (mM) | | | $[Na^+]/[K^+]$ ratio | $Q_{O_2}$ ($\mu$l. of $O_2$/mg. dry wt./hr.) | P/O ratio |
|---|---|---|---|---|---|---|
| | NaCl | KCl | Sucrose | | | |
| Expt. 1 | 7·5 | 12·5 | 260 | 0·6 | 27·9 | 1·2 |
| | 52·5 | 12·5 | 170 | 4·2 | 18·8 | 0·9 |
| | 102·5 | 12·5 | 70 | 8·2 | 10·5 | 0·3 |
| Expt. 2 | 77·5 | 75 | 0 | 1·0 | 10·0 | 0·9 |
| | 77·5 | 20 | 100 | 3·9 | 12·1 | 0·8 |
| | 77·5 | ~0 | 140 | ~50 | 9·4 | 0·3 |
| Means of | 150 | ~0 | — | ~100 | 18·5 | 1·1 |
| several expts. | 140 | 10 | — | 14 | 24·0 | 1·5 |
| | 125 | 25 | — | 5 | 31·0 | 1·6 |
| | 100 | 50 | — | 3 | 33·0 | 1·9 |
| | 50 | 100 | — | 0·5 | 33·0 | 1·9 |
| | 2·5 | 150 | — | 0·02 | 33·0 | 1·9 |

Possible unknown side effects may influence the process under investigation and vitiate their interpretation. Results of these experiments are therefore taken to indicate a trend, and a more precise interpretation of the findings is probably unwarranted, unless supported by other evidence.

Experiments were carried out with mitochondria to see whether the P/O ratio was also dependent on the relative concentrations of $Na^+$ and $K^+$. The results (Table 2) showed essentially the same pattern in the respiration and P/O ratio as observed in the respiration of homogenate. The P/O ratio increased as the $Na^+$ concentration was decreased from 102·5 to 7·5 mM, even though the $K^+$ concentration (12·5 mM) was constant and relatively low. Qualitative agreement with these findings can be seen in results in Fig. 3, in which the concentrations of $Na^+$ and $K^+$ were interchanged, in the absence of osmotic replacements such as choline or sucrose. Compared with observations on homogenates, mitochondrial respiration increased more gradually as the $[Na^+]/[K^+]$ ratio decreased, but the overall picture was the same in the two tissue preparations.

The conclusion that emerges from these experiments is that the increases in P/O ratio of kidney mitochondria observed when increasing concentrations of $K^+$ replace $Na^+$ are due to a specific effect of $K^+$. The magnitude of the effect depends on the $[Na^+]/[K^+]$ ratio and not on the concentration of $K^+$ itself.

*Concentrations of nucleotides and orthophosphate in homogenates*

The concentrations of adenine nucleotides in a respiring homogenate will be determined by several interrelated and often opposing processes, such as $Na^+$-plus-$K^+$-stimulated ATPase activity and the rate and the efficiency of oxidative phosphorylation. Measurements were made of the ATP, AMP and $P_i$ concentrations, in addition to those of ADP, to see whether these were in agreement with those expected from the changes in ATPase activity and P/O ratio induced by $Na^+$, $K^+$ and ouabain. Satisfactory recovery of adenine nucleotides is shown by the results in Table 3. There was a fall in the rate of respiration in the presence of ouabain that was accompanied by falls in the ADP and AMP concentrations and an equivalent rise in the ATP concentration. Very similar results were obtained with malate, and with malate plus glucose. Other preliminary experiments showed that there was no change with time in the concentrations of these nucleotides during the period of incubation of 50 min. under these conditions, when the rate of respiration was steady (Table 3).

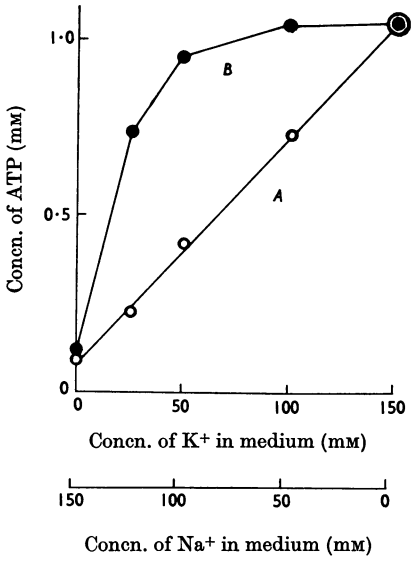


Fig. 4. Effect of $Na^+$, $K^+$ and ouabain on the ATP content of kidney homogenate. Homogenate was incubated for 40 min. as in Fig. 1, with the concentrations of NaCl and KCl indicated. ATP was measured as described in the Methods section. One of five experiments all showing the same result is illustrated. ○, Control; ●, with ouabain (0·25 mM).

*ATP concentration.* Fig. 4 shows the effect of the $Na^+$ and $K^+$ concentrations on the amount of ATP present in the homogenate. When the $Na^+$-plus-$K^+$-stimulated ATPase was inhibited by ouabain, the ATP concentration rose sharply as the $K^+$ concentration was increased, and then flattened out to a constant value. The concentrations of ATP are not related to the ATPase activity of the homogenate, which, in the presence of ouabain, does not vary with $Na^+$ and $K^+$ concentrations. On the other hand, curve *B* in Fig. 4 shows a close resemblance to the change in P/O ratio with similar changes in ionic conditions (see also Fig. 3). It seems likely that the increases in ATP concentration on raising the $K^+$ concentration are due to increases in the rate and efficiency of oxidative phosphorylation. In the homogenate incubated without ouabain, the activity of the $Na^+$-plus-$K^+$-stimulated ATPase would seem to account for the lower ATP concentrations seen when both $Na^+$ and $K^+$ are present (curve *A* in Fig. 4). When only one or other of these ions was added to the homogenates, there was then no effect of ouabain on the ATP concentration.

*AMP concentration.* The effects of $Na^+$ and $K^+$ on the concentration of AMP in the incubated homogenate were the opposite of the effects on ATP concentration. Thus the AMP concentration decreased from about 1 mM in $K^+$-free medium to 0·1 mM at 150 mM-$K^+$ (Fig. 5). Similar results, but with lower absolute concentrations of AMP, were found in the presence of ouabain. Since the formation of AMP probably largely depends on the adenylate-kinase reaction (which is freely reversible and with an equilibrium constant between 0·4 and

Table 3. *Effect of ouabain on nucleotide concentrations in relation to oxygen uptake*

Homogenate was incubated for 50 min. in medium to which glucose and malate were added at a concentration of 10 mM. The KCl and NaCl concentrations were 100 mM and 50 mM respectively, and that of ATP was 1·25 mM. Values quoted are means of the numbers of separate experiments indicated.

| No. of expts. | Substrate | 0·25 mM-Ouabain | $Q_{O_2}$ (μl. of $O_2$/mg. dry wt./hr.) | | Concn. of ATP (mM) | | Concn. of ADP (mM) | | Concn. of AMP (mM) | | Nucleotide recovery (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | − | + | − | + | − | + | − | + | − | + |
| 3 | Glucose + malate | | 21·2 | 14·0 | 0·55 | 0·88 | 0·33 | 0·23 | 0·25 | 0·10 | 90 | 95 |
| 5 | Malate | | 21·8 | 15·7 | 0·60 | 0·97 | 0·33 | 0·20 | 0·28 | 0·09 | 100 | 100 |

1·2; see Bowen & Kerwin, 1956), the AMP concentrations will be determined by the relative concentrations of ATP and ADP. Thus at low $K^+$ concentrations, when the P/O ratio is low and the amount of ATP is low, the adenylate-kinase equilibrium is in favour of AMP.

At high $K^+$ concentrations there is more 'high-energy' phosphate (mainly ATP) in the homogenate, on account of the increased P/O ratio, resulting in the corresponding fall in the AMP concentration shown in Fig. 5. It is difficult to assess in a more quantitative form the outcome of the several different processes, which, combined, ultimately determine the AMP concentration.

*Orthophosphate concentration.* The $P_i$ concentration was found to vary with $Na^+$ and $K^+$ concentrations and with ouabain, in qualitatively the same way as did AMP (Fig. 5), and can be explained by the reasoning applicable to AMP. Comparison of Figs. 4 and 5, with the rate of respiration in Fig. 1, reveals that in no instance is there any correlation such as was found with ADP. Moreover, the addition of $P_i$ (2·5mM) did not alter the pattern of the changes in respiration caused by $Na^+$ and $K^+$, and under these conditions therefore $P_i$ cannot be the factor responsible for respiratory control.

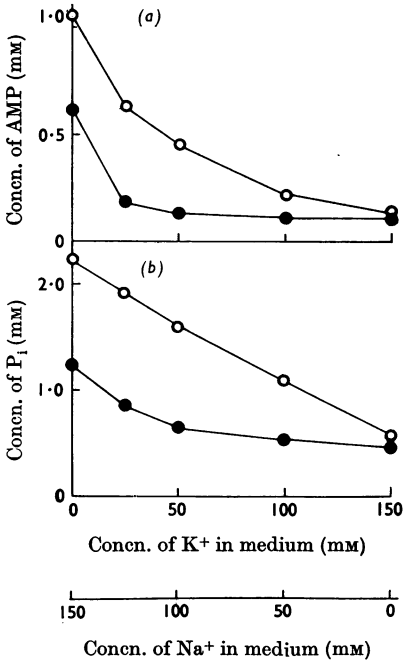


Fig. 5. Effect of $Na^+$, $K^+$ and ouabain on the AMP and $P_i$ content of kidney homogenate. Experimental conditions were as described in Fig. 4. (*a*) ○, AMP control; ●, AMP with ouabain (0·25mM). (*b*) ○, P control; ●, $P_i$ with ouabain (0·25mM).

## DISCUSSION

The results described above with homogenates and mitochondria throw light on the mechanism of the control of respiration by $K^+$ on tissue slices and in the intact organ. There are two effects, one direct on mitochondria, and the other indirect through an action on the cell-membrane ATPase. The latter effect is closely related to the amount of active cation-transport work being done. Thus there is an increase in oxygen consumption of tissue slices or in the intact kidney associated with active transport (Whittam, 1961; Lassen, Munck & Thaysen, 1961; Burg & Orloff, 1962; Whittam & Willis, 1963). Analysis of ATPase activity cannot be made in tissue slices, but in homogenates there is an increase in this activity that causes proportional changes in the oxygen uptake (Blond & Whittam, 1964*a*). It has now been shown (Fig. 2) that the variations in oxygen uptake are correlated with changes in ADP concentration.

The present results may be compared with the earlier work of Chance & Williams (1955, 1956) and Chance & Hagihara (1961) on the control of mitochondrial respiration by ADP. The relative increases in oxygen uptake associated with a given relative increase in ADP concentration are of the same order of magnitude in our work with homogenates and in the work with isolated mitochondria. Nevertheless, our results differ quantitatively in showing proportionality of oxygen uptake and ADP concentration over the range 100–400 $\mu$M-ADP, whereas with mitochondria the range 10–40 $\mu$M-ADP was found. A comparable discrepancy has been reported by Williamson (1965), who found that the elevated oxygen uptake of the perfused rat heart, on stimulation with epinephrine, is associated with an increase in ADP concentration from approx. 1 to 2mM. Respiration is stimulated by an increase of mitochondrial ADP, whereas the net changes found with kidney homogenates and the intact heart represent an overall change in the intra- and extra-mitochondrial components. Nonetheless, the nucleotide concentrations in our experiments were constant during the 30–60min. period when the oxygen uptake was measured, and the different rates of respiration were associated with different and unchanging ADP concentrations. It is an open question how the quantitative discrepancy with mitochondrial work arises. An important factor may be the $[ADP][P_i]/[ATP]$ ratio, as shown by the work of Klingenberg & Schollmeyer (1960, 1961). Another point is that the type of respiratory substrate used might affect the ADP concentration that is required for respiratory control. Preliminary experiments with substrates other than malate have indicated considerable differences in the changes in the

nucleotide concentrations associated with respiratory changes caused by $Na^+$, $K^+$ and ouabain.

The bearing of the present results on respiratory control *in vivo* rests on the validity of attributing to the components of cells in homogenates the same kinds of properties that they possessed in the intact cell. This appears to be reasonably valid, but a note of caution is necessary inasmuch as work with intact kidneys may present a more complex picture. Measurements of $Na^+$ reabsorption and oxygen consumption of dog kidney showed that, although both processes were depressed by cyanide, reabsorption continued when oxygen consumption was increased by the action of 2,4-dinitrophenol (Fujimoto, Nash & Kessler, 1964). A fall in ATP production would be expected in these circumstances, and it has been suggested that energy for $Na^+$ reabsorption *in vivo* may come directly from oxidative metabolism without the involvement of ATP. On the other hand, the present work agrees with that of Aubert, Chance & Keynes (1964) and Maitra, Ghosh, Schoener & Chance (1964), who examined the sequence of events after stimulation of the electric organ of the electric eel (*Electrophorus electricus*). Increased rates of $Na^+$ extrusion that follow electrical stimulation caused a rise in the concentrations of ADP, AMP and $P_i$. In electric tissue, as in kidney cortex, there is direct evidence that a control mechanism exists such that products of energy utilization stimulate energy production. Measurements of nucleotide concentrations do not appear to have been made with other cells and tissues in relation to ATPase activity. There is circumstantial evidence, however, that liver, salt gland, brain cortex, thyroid gland, striated muscle, frog skin, toad bladder and erythrocytes fit into the same picture. It appears that glycolysis and respiration alike, as sources of energy for cells, can be regulated accordingly to the extent of ATP utilization for active transport.

The respiration of kidney homogenates and mitochondria is influenced by $Na^+$ and $K^+$ directly, as well as indirectly as described above. Thus, with pure mitochondria, or in homogenates containing ouabain, there is still stimulation of respiration by $K^+$. This is not associated with increased ATPase activity. With less than 50mM-$K^+$, the rate of respiration and the P/O ratio of mitochondria fell (Table 1). As regards $Na^+$, this ion counteracted the mitochondrial stimulation by $K^+$, in contrast with its synergic effect with $K^+$ on the indirect control through the cell-membrane ATPase. There seem to be direct effects of $Na^+$ and $K^+$ on mitochondria. $K^+$ apparently increases the respiratory response to ADP by enhancing the tightness of coupling of electron transport and oxidative phosphorylation in the respiratory chain. The P/O ratio was almost doubled on raising the $K^+$ concentration from 0 to 50mM. The $K^+$ concentration in the kidney *in vivo*, and in slices incubated *in vitro* under optimum conditions, is 70–80 $\mu$equiv./g. of tissue, suggesting that the $K^+$ concentration is normally sufficiently high to ensure a maximum P/O ratio. In slices with a low $K^+$ content, it would seem that the P/O ratio may be low.

A possible alternative cause of the effect of $K^+$ is that its accumulation by mitochondria, which is an energy-dependent process (Gamble, 1957), might result in an increased rate of respiration. This is an unlikely explanation, first, because $K^+$ concentrations that stimulate oxygen uptake do not increase the rate of ATP hydrolysis by mitochondria (Blond & Whittam, 1964*a*), and secondly, because the respiratory response to $K^+$ is counteracted by $Na^+$ (Table 2), whereas the uptake and binding of $K^+$ is unaffected by $Na^+$ (Stanbury & Mudge, 1953; Berger, 1957; Gamble, 1957).

Although removal of $K^+$ from the medium causes a fall in the P/O ratio, this is associated with a fall in the respiration (Fig. 1). Uncoupling agents, such as 2,4-dinitrophenol, on the contrary, cause increased rates of respiration. This difference can be explained if $K^+$ is necessary to obtain optimum rates of either oxidation or phosphorylation, in addition to its role in promoting tight coupling of these processes. Pressman & Lardy (1955), using rat-liver mitochondria, showed that, when respiration was stimulated by hexokinase and glucose, microsomes, or low concentrations of 2,4-dinitrophenol, a requirement for $K^+$ became apparent. They concluded that the need for $K^+$ for oxidative phosphorylation was satisfied by the endogenous $K^+$ when rates of oxidative phosphorylation were low, as in the unstimulated system. These authors also reported that, when respiration and phosphorylation were uncoupled by 2,4-dinitrophenol, the need for $K^+$ disappeared, indicating that $K^+$ was needed for phosphorylation reactions rather than for oxidative processes. Krall, Wagner & Gozansky (1964) reported that oxidative phosphorylation in brain mitochondria is stimulated by $K^+$.

A new observation on the $K^+$ requirement of mitochondria for oxidative phosphorylation is that the absolute $K^+$ concentration is not the determining factor, but that it is rather the relative concentrations of $K^+$ and $Na^+$. Thus maximum P/O ratios are found with $K^+$ concentrations as low as 12·5mM, provided that the concentration of $Na^+$ is also low (Table 2). It appears from this that $Na^+$ might be competing with $K^+$ for a site controlling a reaction that is stimulated by $K^+$ only. Reactions catalysed by $K^+$ and inhibited by $Na^+$ are known, e.g. the phosphorylation of ADP by phosphoenolpyruvate (Boyer, Lardy & Phillips, 1943; Utter, 1950). Reactions involving transfer

of 'high-energy' phosphate in the mitochondria might well therefore be the reactions through which $K^+$ exerts its effect. Our results do not exclude the possibility that the absence of $K^+$ causes damage to mitochondrial membranes that might account for their impaired function. Omission of $K^+$ is known to modify considerably the permeability of mitochondria (Amoore, 1960). It seems likely that, under some conditions, respiration is limited by the entry of ADP into mitochondria, and not simply by the concentration of extramitochondrial ADP (see Siekevitz & Potter, 1955), and $K^+$ may exert an effect on this process also.

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