Respiratory Control by an Adenosine Triphosphatase Involved in Active Transport in Brain Cortex

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The immediate source of energy for the nerve impulse is the movement of Na^+ and K^+ ions down their concentration gradients, and one of the main roles of metabolism in nervous tissue is to provide energy for the transfer of these ions in the reverse direction during the recovery process. The dependence of active transport in nervous tissue on a supply of energy from respiration is now well established (Turner, Eggleston & Krebs, 1950; Hodgkin & Keynes, 1955; Pappius & Elliott, 1956), and high-energy phosphate compounds, especially ATP, seem to be the immediate sources of energy. The converse of the dependence of active transport on a supply of energy from respiration is the regulation of respiration by the energy demands of active transport, so that the production of energy-rich compounds occurs at a rate commensurate with their rate of utilization in ion transport. This has been demonstrated in brain and kidney-cortex slices, in which changes in the rate of uptake of K^+ ions, induced by specific means that were believed not directly to inhibit respiration, are accompanied nevertheless by parallel changes in respiration (Whittam, 1961, 1962a; Whittam & Willis, 1963). It was therefore suggested that active cation transport could act as a pacemaker for about 40 % of the respiration of the cell. However, there was no direct evidence on the mechanism by which the rate of ion transport, occurring at the cell membrane, controlled the rate of cellular respiration which is exclusively located in the mitochondria.

How does an event at the cell membrane affect events in the mitochondria? A possible approach to this problem was suggested by the finding in many tissues of an adenosine triphosphatase that is activated by Na^+ and K^+ ions and inhibited by ouabain. The quantitative resemblance of the properties of the adenosine triphosphatase with those of the coupled transport of Na^+ and K^+ ions has led to the general belief that the two processes are aspects of the same mechanism (Skou, 1957, 1962; Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961; Wheeler & Whittam, 1962). This idea is further supported by the localization of the Na^+ ion-plus- K^+ ion-activated adenosine-triphosphatase activity in the neuronal membrane (Cummins & Hyden, 1962), and the fact

that the adenosine-triphosphatase activity in the erythrocyte stroma is stimulated by internal Na^+ ions and external K^+ ions, these being the locations from which the ions are actively transported (Whittam, 1962b; Glynn, 1962). These observations therefore raise the possibility that the adenosine triphosphatase associated with active transport might be the means by which the latter controls the rate of respiration in slices. This would be feasible on mechanistic grounds, by analogy with the control of the respiration of isolated mitochondria by the availability of ADP or orthophosphate, both of which would be products of ATP hydrolysis (Lardy & Wellman, 1952).

In the present study, the characteristics of respiration and adenosine-triphosphatase activity have been compared under similar conditions of incubation. The results show that there is a parallelism between the activity of the Na^+ ion-plus- K^+ ion-activated adenosine triphosphatase and a part of the respiration, suggesting that the rate of energy production by respiration is geared to its rate of utilization.

METHODS

Preparation of tissue suspensions. Adult rabbits of both sexes were stunned by a blow on the neck, bled from the throat and decapitated. The brain was excised and most of the tissue other than the cortex was cut away. About 5 g. of the cortex with some adhering white matter was then homogenized in about 12 ml. of ice-cold sucrose solution (0.25 M) in a Potter-Elvehjem-type homogenizer with a smooth glass tube and Teflon pestle (total clearance 0.01 in.; 700 rev./min.). The pestle was taken six times up and down through the suspension over a total period of 2 min. Doubling the time and extent of homogenization was without effect on the respiration of the homogenate under various conditions, indicating that any effects observed under these conditions were not due to the presence of intact cells. The homogenate was strained through muslin and the volume made to 15 ml. by the addition of more sucrose solution.

Preparation of mitochondria and microsomes. Mitochondria were prepared by the following procedure except when described otherwise. A 10 % (w/v, referred to wet wt. of tissue) suspension of homogenate in 0.25 M-sucrose was centrifuged at 1500g for 10 min. at 0° (MSE Major refrigerated centrifuge, 6886 head; 3200 rev./min.) to remove nuclei and cell debris. The sediment was discarded, as was

that obtained after centrifuging the supernatant again at 1500g for 10 min. Mitochondria were prepared by further centrifuging at 17 000g for 10 min. (MSE Major refrigerated centrifuge, superspeed attachment; 16 500 rev./min.). The sediment was resuspended in 0.25M-sucrose by gentle homogenization, the volume made to about 6 ml. and the suspension recentrifuged at 17 000g for 10 min. The mitochondrial pellet was suspended in about 7.5 ml. of medium A, containing NaCl (130 mM), MgSO_4 (1.3 mM), nicotinamide (22.5 mM) and potassium phosphate buffer, pH 7.5 (6 mM).

On account of the time required for their preparation, microsomes were prepared from the mitochondrial supernatant that had been stored for 2 days at 0–4°. The few remaining mitochondria were first removed by a preliminary spin at 20 000g for 10 min. and the supernatant was then centrifuged at 104 000g for 60 min. in the no. 40 head of the Spinco model L preparative ultracentrifuge. The straw-coloured gelatinous sediment was resuspended in 3–4 ml. of 0.25M-sucrose and used without further washing.

Measurement of respiration. Portions of homogenate (1.0 ml.; 50–60 mg. dry wt.) were added to manometer cups containing 3.0 ml. of medium, and the O_2 consumption was measured during incubation at 37° in air. Incubation commenced usually about 30 min. after removal of the brain from the animal and was continued for 40–60 min., during which time the rate of respiration was linear. The incubation medium contained (final concentrations) glucose (10 mM), imidazole-HCl buffer, pH 7.5 (15 mM), MgCl_2 (2 mM), and various concentrations of NaCl and KCl, the sum of which was constant at 150 mM.

The rate of respiration of mitochondria was not linear in this medium, and fell off rapidly during incubation. A medium (medium B) containing MgSO_4 (8.5 mM), potassium phosphate buffer, pH 7.5 (14 mM), tris-HCl buffer, pH 7.5 (14 mM), EDTA (0.4 mM), nicotinamide (36 mM), glucose (14 mM), sodium pyruvate (14 mM), potassium malate (7 mM), ATP (disodium salt) (0.8 mM), NAD (0.7 mM) and NaCl (75 mM), based on that described by Balazs (1959), was found to support a linear rate of O_2 uptake for incubation periods of 60 min. and was therefore used in subsequent experiments. The final concentrations of Na^+ and K^+ ions due to media A and B were 126 mM- Na^+ ion and 47 mM- K^+ ion. The oxygen consumption of mitochondria (0.5 ml.; 10–15 mg. dry wt.) was measured in air at 37°, and commenced 2–2.5 hr. after the removal of the brain from the animal.

Adenosine-triphosphatase activity. Reaction mixtures (1.9 ml.) contained (final concentrations) imidazole-HCl buffer, pH 7.5 (15 mM), MgCl_2 (1.25 mM), various amounts of the ions under investigation and the tissue suspension (dry wt. 0.2–0.5 mg. of homogenate, 0.2–0.4 mg. of mitochondria). The tubes were shaken in a water bath at 37° for 3 min. and the reaction was initiated by the addition of 25 mM-ATP (0.1 ml.; disodium salt buffered to pH 7.5 with tris) to give a final concentration of 1.25 mM, which was above that of the K_m for ATP, measured in separate experiments. After 3 min. the reaction was stopped by adding 0.5 ml. of ice-cold 35% (w/v) trichloroacetic acid, and the tubes were placed in ice and water for about 15 min. After the precipitated protein had been spun down, orthophosphate was measured in samples from the supernatants by the method of Fiske & Subbarow (1925). Adenosine-triphosphatase

activity is expressed as μmoles of orthophosphate released/mg. dry wt./hr. The assay was linear up to 15 min. incubation time, and linear with increasing tissue weight up to 0.25 mg. dry wt./ml.

The accuracy of the measurements of the rates of hydrolysis of ATP in a single experiment was estimated by measuring six times the amounts of orthophosphate produced during incubation in the presence and absence of 100 μM -ouabain, and in Na^+ ion-rich and Na^+ ion-free media. The media contained NaCl (150 mM) or choline chloride (150 mM), KCl (5 mM), MgCl_2 (1.25 mM) and ATP (1.25 mM). The mean rates $\pm$ s.e.m. of orthophosphate liberation (μmoles of orthophosphate/mg. dry wt./hr.) were 6.0 ± 0.1 (range 5.9–6.2) in the control, 4.4 ± 0.1 (range 4.3–4.5) with ouabain and 4.5 ± 0.1 (range 4.4–4.6) in the absence of Na^+ ions.

Paper chromatography. To determine the product of the hydrolysis of ATP, samples of the supernatant solutions remaining after precipitation of the protein were analysed qualitatively for ATP, ADP and AMP by paper chromatography according to the method of Krebs & Hems (1953).

Products of hydrolysis of adenosine triphosphate. The trichloroacetic acid extracts from the preliminary experiments were analysed for ATP, ADP and AMP by paper chromatography, and the papers photographed in ultraviolet light. Under the conditions chosen, only ADP besides ATP could be detected, and the amounts of ADP increased as the incubation time was lengthened or the tissue concentration increased. The standard conditions given above therefore provide a means of measuring the rate of breakdown of ATP to ADP.

Tissue concentration. The dry weight of the tissue suspension was obtained by drying in an oven at 105° overnight. When determining the adenosine-triphosphatase activities of subcellular fractions, the protein content of the suspension was used as a basis for comparison of the rates of ATP hydrolysis. Protein was estimated by the biuret method (Robinson & Hogden, 1940; Layne, 1957). A modification was introduced to extract lipid from the tissue to avoid opalescence in the final coloured solution. A sample of tissue suspension was deproteinized with trichloroacetic acid and the precipitate spun down. The supernatant was decanted and the sediment resuspended in 10 ml. of ethanol-diethyl ether (13:7, v/v) mixture and allowed to stand for 1 hr. The precipitate was spun down and dissolved in 4% (w/v) NaOH (1.0 ml.) by heating at 100° for 5 min. The biuret reagent (4.0 ml.) was then added and the solution left at room temperature for 30 min., before measurement of the extinction at 555 m μ . Standard solutions, prepared from crystalline bovine plasma albumin, reagent blanks and sucrose blanks were treated in the same way. The protein content of tissue fractions is expressed as their equivalent of albumin.

Materials. Ouabain (strophanthin-G), imidazole, choline chloride, nicotinamide, trichloroacetic acid and pyruvic acid were laboratory-reagent products of British Drug Houses Ltd., Poole, Dorset; hexokinase and NAD were supplied by Boehringer und Soehne G.m.b.H., Mannheim, Germany; L-malic acid was a product of L. Light and Co. Ltd., Colnbrook, Bucks.; bovine plasma albumin (fraction V) was obtained from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex; ATP (disodium salt) and tris (Sigma 7-9) were from the Sigma Chemical Co., St Louis, Mo., U.S.A.; all other compounds used were AnalaR reagents

from British Drug Houses Ltd. Malic acid and pyruvic acid were used as their potassium and sodium salts respectively. All solutions were made in glass-distilled water.

RESULTS

Respiration of brain homogenates

To examine the dependence of respiration on the concentrations of Na^+ and K^+ ions, homogenates were incubated with various amounts of these ions. Fig. 1 shows that when the sum of the concentrations of Na^+ and K^+ ions was kept constant at 150 mM, maximum respiration occurred with 0–50 mM- K^+ ions, and with the Na^+ ion concentration 100–150 mM. As Na^+ ions were further replaced by K^+ ions, respiration decreased, until at 150 mM- K^+ ion the Q_{O_2} had fallen from 3.2 to 2.4. The magnitude of the decrease varied somewhat in different experiments, but was usually about 30%. Replacement of 150 mM- Na^+ ion with choline at a K^+ ion concentration of 5 mM decreased the Q_{O_2} from 2.9 (s.e.m. ± 0.10 ; $n = 9$) to 1.8 (s.e.m. ± 0.11), a decrease of about 40%. From these results it appears that concentrations of Na^+ ions greater than 50 mM are essential for maximum rates of respiration. The respiration remained constant as the concentration of added K^+ ions was decreased to zero, suggesting that any requirement for K^+ ions for maintenance of maximum rates of respiration is satisfied by the K^+ ions supplied in the homogenate itself, which would give a final concentration of about 6 mM.

Fig. 1. Respiratory response of brain homogenates to Na^+ ions, K^+ ions and ouabain. Samples (1.0 ml.) of homogenate were incubated for 40 min. at 37° in 3.0 ml. of medium containing (final concentrations) imidazole-HCl buffer, pH 7.5 (15 mM), glucose (10 mM), MgCl_2 (2 mM), and the concentrations of Na^+ and K^+ ions indicated, which include 6 mM- K^+ ion and 4 mM- Na^+ ion from endogenous sources. ○, Control; ●, with 0.1 mM-ouabain. The Figure illustrates a single experiment representative of five such experiments.

Ouabain caused a fall in the maximum rate of respiration with optimum concentrations of Na^+ and K^+ ions, from Q_{O_2} 3.2 to 2.4. Respiration in the presence of ouabain was independent of the concentrations of Na^+ and K^+ ions, and the Q_{O_2} remained constant at 2.4, identical with that found on replacement of Na^+ ions with K^+ ions or choline. The magnitude of the inhibition by ouabain with optimum concentrations of Na^+ and K^+ ions varied somewhat, but in nine experiments the Q_{O_2} was decreased about 30%, from 2.9 (s.e.m. ± 0.10) to 2.0 (s.e.m. ± 0.11). The results suggest that the total respiration is controlled by two factors, one of which is the site of action of both ouabain and Na^+ ions.

Adenosine-triphosphatase activity

The rate of ATP hydrolysis was measured to see whether the effects of cations and ouabain on respiration might be mediated by adenosine triphosphatase sensitive to these agents.

Sensitivity of adenosine-triphosphatase activity to Na^+ ions, K^+ ions and ouabain. The influence of Na^+ ions, K^+ ions and ouabain on the rate of ATP hydrolysis is shown in Fig. 2. Adenosine-triphosphatase activity was stimulated only when Na^+ and K^+ ions were present together. Maximum activity occurred with 30–50 mM- K^+ ion plus 120–100 mM- Na^+ ion, and was about 40% greater than that with 150 mM- Na^+ ion or with 150 mM- K^+ ion plus 2.5 mM- Na^+ ion. The increase in adenosine-triphosphatase activity was much steeper when the K^+ ion concentration in the medium was increased from zero than when the Na^+ ion concentration was similarly increased, indicating that smaller amounts of K^+ ions than Na^+ ions were needed to stimulate activity. Replacement of Na^+ ions with choline decreased the activity to that found when either Na^+ or K^+ ions were absent. A fall to the same activity was found on the addition of ouabain, at various concentrations of Na^+ and K^+ ions. Ouabain thus appears specifically to inhibit the adenosine-triphosphatase activity that is stimulated by Na^+ and K^+ ions.

The results suggest that, from the point of view of the effects of Na^+ ions, K^+ ions and ouabain, adenosine-triphosphatase activity, like respiration, can be regarded as being composed of two components. One is stimulated by the combined presence of Na^+ and K^+ ions and inhibited by ouabain, and is responsible for 30–40% of the total activity. The other component is insensitive to these ions and to ouabain.

Correlation of respiration and adenosine-triphosphatase activity in brain homogenate

The question of the identity of the Na^+ ion-plus- K^+ ion-activated adenosine triphosphatase with the

Na⁺ ion-plus-K⁺ ion-dependent factor that controls respiration has been investigated by making parallel measurements of the inhibition of each by increasing concentrations of ouabain.

The inhibition of the rates of respiration and ATP hydrolysis by increasing concentrations of ouabain is shown in Table 1. In Expt. 1 the Q_{O_2} of homogenate incubated with 100 μ M-ouabain fell from 3.3 (the control value) to 2.1, a decrease of 37%. A partial inhibition of respiration was produced by lower concentrations of ouabain with half-maximum inhibition occurring at 2.5–5.0 μ M-ouabain, which also gave half-maximum inhibition of adenosine-triphosphatase activity. The maximum decrease in the rate of ATP hydrolysis (μ moles of orthophosphate liberated/mg. dry wt./hr.) was from 5.7 in the control to 3.3 with 100 μ M-ouabain. The ouabain-sensitive adenosine-triphosphatase activity was thus 42% of the total. The results of Expt. 2 in Table 1 show a similar effect. To compare the relative effects of ouabain on Q_{O_2} and the rate of ATP hydrolysis, the results in Table 1 have been replotted as the extent of inhibi-

tion of adenosine-triphosphatase activity against inhibition of respiration by the same concentrations of ouabain (Fig. 3). The linearity of the plot indicates identical relative inhibitions of Q_{O_2} and adenosine-triphosphatase activity by different concentrations of ouabain. Any effect on one process was accompanied by an equivalent effect on the other, suggesting coupling of the two processes.

Effects of Ca²⁺ ions and ethylenediaminetetra-acetic acid on respiration and rate of adenosine triphosphate hydrolysis

Since Ca²⁺ ions cause marked changes in adenosine-triphosphatase activity and on the respiration of brain slices, the effects of Ca²⁺ ions on these processes in brain homogenates has been examined.

Effect of Ca²⁺ ions on respiration. Incubation of homogenates in the presence of increasing concentrations of Ca²⁺ ions resulted in a progressive inhibition of respiration which increased to 35–40% at 10 mM-Ca²⁺ ion, when the Q_{O_2} had fallen from 3.8 to

Fig. 2. Stimulation by Na⁺ and K⁺ ions of the adenosine-triphosphatase activity in brain-cortex homogenate. Samples of homogenate (0.4 mg. dry wt.) were incubated for 3 min. in a reaction mixture containing imidazole-HCl buffer, pH 7.5 (15 mM), MgCl₂ (1.25 mM), ATP (disodium salt, buffered to pH 7.5 with tris) (1.25 mM), and the concentrations of Na⁺ and K⁺ ions indicated. The Na⁺ ions added in the ATP solution have been included in the concentration shown on the abscissa. The reaction was stopped by adding 0.5 ml. of 35% (w/v) trichloroacetic acid, and the precipitated protein spun down. Samples from the supernatant were analysed for orthophosphate by the method of Fiske & Subbarow (1925). ○, Control; ●, with 0.1 mM-ouabain; △, choline in place of Na⁺ ions. All points are means of duplicates which agreed to within 5%. The experiment is representative of a group of three similar experiments.

Table 1. *Inhibition by ouabain of respiration and adenosine-triphosphatase activity in brain-cortex homogenate*

Q_{O_2} consumption was measured on samples of homogenate incubated in the presence of glucose (10 mM), imidazole-HCl buffer, pH 7.5 (15 mM), MgCl₂ (2 mM), KCl (5 mM), NaCl (150 mM) and ouabain at the concentrations indicated. Adenosine-triphosphatase activity was measured in the same homogenate after diluting tenfold with 0.25M-sucrose. The reaction mixture contained (final concentrations) ATP (disodium salt, buffered to pH 7.5 with tris) (1.25 mM), MgCl₂ (1.25 mM), imidazole-HCl buffer, pH 7.5 (10 mM), KCl (5 mM) and NaCl (150 mM), with various concentrations of ouabain. The amount of orthophosphate liberated was measured after incubation for 3 min. at 37°. Each value is the mean of duplicate determinations.

Concn. of ouabain (μ M)	Rate of ortho-phosphate liberation (μ moles/mg. dry wt./hr.)	Q_{O_2} (μ l. of O_2 /mg. dry wt./hr.)	Percentage decrease due to ouabain	
			Q_{O_2}	Ortho-phosphate liberation
Expt. 1				
0	5.7	3.3	—	—
0.625	5.2	3.2	4	9
1.25	5.0	3.1	7	12
2.5	4.7	2.7	16	17
5.0	4.2	2.5	26	26
10.0	3.7	2.3	32	35
100	3.3	2.1	36	42
Expt. 2				
0	5.5	3.2	—	—
0.625	5.1	3.0	6	7
1.25	4.9	2.9	10	11
2.5	4.6	2.7	16	16
5.0	4.1	2.4	25	26
10.0	3.8	2.2	31	31
100	3.2	2.0	39	42

2.4 (Fig. 4). This inhibition was less marked if ouabain was also present, when 10 mM- Ca^{2+} ion decreased the Q_{O_2} only 20 %, from 2.9 to 2.25. The ouabain-sensitive part of the respiration appears to be particularly susceptible to inhibition by Ca^{2+} ions, since it was decreased 90 % by 10 mM- Ca^{2+} ion, when the ouabain-insensitive component of respiration was decreased only 20 %.

Effect of Ca^{2+} ions on adenosine-triphosphatase activity. The rates of ATP hydrolysis were measured

Fig. 3. Parallelism of the graded inhibition of respiration and adenosine-triphosphatase activity in brain-cortex homogenates, by different concentrations of ouabain. Each point is the mean of four determinations made in two separate experiments.

Fig. 4. Inhibition by Ca^{2+} ions of the respiration of brain homogenate. Samples of homogenate (1.0 ml.) were incubated for 60 min. at 37° in a medium (3.0 ml.) containing (final concentrations) glucose (10 mM), MgCl_2 (2 mM), imidazole-HCl buffer, pH 7.5 (20 mM), KCl (25 mM), NaCl (120 mM) and the concentrations of CaCl_2 indicated. $\circ$, Control; $\bullet$, with 25 μM -ouabain.

at different concentrations of Ca^{2+} ions, and the results (Fig. 5) show that Ca^{2+} ions in the concentration range 0.001–1.0 mM inhibited adenosine-triphosphatase activity in the presence and absence of ouabain. The extent of the inhibition was proportional to the logarithm of the Ca^{2+} ion concentration. In the presence of ouabain the inhibition by Ca^{2+} ions was diminished, for 1 mM- Ca^{2+} ion caused a 75 % fall in the rate of ATP hydrolysis in the absence of ouabain, and only a 40 % fall in its presence; the decrease in the ouabain-sensitive part on the addition of 1 mM- Ca^{2+} ion was 84 %, from 4.5 to 0.75. Again, Ca^{2+} ions inhibited both the ouabain-insensitive and ouabain-sensitive components of adenosine-triphosphatase activity, but the latter was inhibited to a much greater relative extent. The fall in activity of the ouabain-sensitive part of the adenosine triphosphatase was nearly twice that of the ouabain-insensitive component. The parallel between this and the greater sensitivity to Ca^{2+} ions of the ouabain-sensitive component of respiration is striking, and suggests again that the two processes may be closely connected.

Effect of ethylenediaminetetra-acetic acid on adenosine-triphosphatase activity and respiration. Since the addition of Ca^{2+} ions inhibits the rates of ATP hydrolysis and of respiration it was decided to

Fig. 5. Inhibition by Ca^{2+} ions of the adenosine-triphosphatase activity of brain-cortex homogenate. Samples of homogenate were incubated for 5 min. at 37° in a medium containing (final concentrations) imidazole-HCl buffer, pH 7.5 (15 mM), MgCl_2 (1.25 mM), ATP (disodium salt, buffered to pH 7.5 with tris) (1.25 mM), KCl (20 mM), NaCl (120 mM), various concentrations of CaCl_2 and 0.22 mg. dry wt. of tissue. $\circ$, Control; $\bullet$, with 50 μM -ouabain. The Figure illustrates a single experiment typical of four similar experiments.

see whether the removal by EDTA of the endogenous Ca^{2+} ions in the tissue led to increases in the rates of these processes.

A range of EDTA concentrations from 0.005 to 5 mM was added to incubation mixtures and their effect on adenosine-triphosphatase activity is shown in Fig. 6. There was a twofold increase in the rate of ATP hydrolysis with 5–100 μM -EDTA which was abolished if the Na^+ ions in the medium were replaced by choline or if ouabain was added. At concentrations 5–100 μM -EDTA appears therefore to stimulate specifically the Na^+ ion- and ouabain-sensitive adenosine-triphosphatase activity. Higher concentrations of EDTA (0.1–5 mM) caused a pronounced decrease in activity, regardless of the Na^+ ion content of the medium or the presence of ouabain, until at 5 mM-EDTA adenosine-triphosphatase activity had fallen to zero. Thus EDTA at high concentrations inhibited both components of the adenosine-triphosphatase activity. The results show that the part of the adenosine-triphosphatase activity affected by low concentrations of EDTA is that which is sensitive to Na^+ ions and ouabain. It is also this component that is particularly sensitive to inhibition by Ca^{2+} ions (Fig. 5), suggesting that the stimulation of activity by EDTA may be the result of removal of small amounts of Ca^{2+} ions

Fig. 6. Effect of EDTA on the adenosine-triphosphatase activity of brain-cortex homogenate. Various concentrations of EDTA were added to tissue suspensions containing (final concentrations) imidazole-HCl buffer, pH 7.5 (15 mM), MgCl_2 (1.25 mM), ATP (disodium salt, buffered to pH 7.5 with tris) (1.25 mM), KCl (20 mM), NaCl (120 mM) or choline chloride (120 mM) and 0.22 mg. dry wt. of tissue. The amount of orthophosphate liberated was determined after incubating at 37° for 5 min. $\circ$, Control; $\bullet$, with 50 μM -ouabain; $\triangle$, choline in place of Na^+ ions. The Figure illustrates a single experiment typical of a group of five such experiments.

present in the tissue. The inhibition of ATP hydrolysis at higher EDTA concentrations was probably the result of gradual chelation of Mg^{2+} ions, thereby preventing the formation of the Mg^{2+} ion-ATP complex necessary for enzymic hydrolysis of ATP.

The respiratory response of homogenates to EDTA, although somewhat variable, indicated that EDTA at concentrations up to 5 mM had little effect on respiration. An explanation of this is difficult in view of the complexity of the possible interactions of EDTA with the respiratory system.

Location of the site of action of ouabain on respiration and adenosine-triphosphatase activity

The effect of ouabain has been examined on the respiration of a mitochondrial fraction, and on the adenosine-triphosphatase activity of various sub-cellular fractions obtained by differential centrifugation of the whole homogenate.

Respiration of mitochondria. The action of ouabain on the respiration of mitochondrial fractions prepared in different ways shows that Q_{O_2} was lowered 24–42 %, according to the method of preparation (Table 2). Collection of the mitochondrial fraction at 200 000 g-min., after removal of nuclei and debris at 20 000 g-min., gave a preparation whose respiration was depressed 42 % by ouabain. When the mitochondrial fraction was collected at lower centrifugal forces, and washed twice, the inhibition was

Table 2. *Inhibition by ouabain of the respiration of mitochondrial preparations from brain cortex*

Nuclei and cell debris were removed from a 10 % (w/v) brain homogenate by centrifuging at 2000 g for 10 min. and discarding the sediment. Mitochondria were prepared from the supernatant at the centrifugal forces indicated. After decanting the supernatant, the tubes were drained, the mitochondrial pellets were resuspended in 0.25 M-sucrose by gentle homogenization and the volume was made up to 40 ml. with 0.25 M-sucrose. The mitochondria obtained by recentrifuging the resulting suspension at the appropriate speed were either washed once again in this way, or resuspended in medium A (see the text) and the volume was made up to 7.5 ml. Portions (0.5 ml.) of the mitochondrial suspension were incubated in medium B (see the text) and the O_2 uptake at 37° was measured over a period of 40 min. The results refer to three separate experiments.

Fraction separated	No. of times washed	O_2 uptake ($\mu\text{l. of O}_2/\text{hr.}$)		Percentage decrease due to ouabain
		Control	With ouabain	
At 20 000 g for 10 min.	1	285	163	42
At 16 000 g for 10 min.	2	225	163	26
At 12 000 g for 10 min.	2	115	88	24

decreased to 24 %. This difference in sensitivity implies that part of the ouabain inhibition of the more sensitive preparation was due to contamination by particles less easily sedimented than mitochondria. The number of washings of the mitochondrial pellet was further increased to obtain a purer mitochondrial fraction, but the mitochondria then lost the ability to respire at a constant rate, if at all. It remains uncertain, therefore, whether inhibition of mitochondrial respiration by ouabain is due to contamination by extramitochondrial particles that are sensitive to ouabain and that influence the rate of respiration, or whether ouabain has a direct effect on mitochondria.

Adenosine-triphosphatase activity of subcellular fractions. Brain-cortex homogenate was separated into seven fractions, each of which was examined under the phase-contrast microscope. The main components observed are noted in Table 3. The appearance of the mitochondrial fraction sedimented at 10 000g or at 16 000g (fractions 3 and 4) was that of a fairly homogeneous suspension, but the presence of some microsomes could not be excluded, since even in a concentrated suspension the latter were barely visible as discrete particles. Other fractions consisted largely of nuclei (fraction 1), mitochondria and nuclei (fraction 2), mitochondria and microsomes (fraction 5), and microsomes

(fraction 6). The protein content of the whole homogenate and the various fractions was determined: 80 % of the total protein was recovered in the fractions.

With the exception of the supernatant, each fraction possessed, in some degree, adenosine-triphosphatase activity sensitive to ouabain (Table 3). The complete absence in the supernatant of ouabain-sensitive adenosine triphosphatase suggests that this is a property associated with particulate matter. The specific activity (μ moles of orthophosphate released/mg. of protein/hr.) of the ouabain-sensitive adenosine-triphosphatase activity was 6.4 in both the nuclear fraction and in the whole homogenate, and was somewhat variable (3.2-7.4) in the mitochondrial fractions. In contrast, the specific activity of the microsomes was much greater (16.1). The absolute activity of the ouabain-sensitive microsomal adenosine triphosphatase was 45 % of the total ouabain sensitivity and 17 % of the total adenosine-triphosphatase activity. A significant proportion of the total activity is thus localized in the microsomal fraction. The results do not show whether the ouabain sensitivity found in particulate fractions other than microsomal was due to contamination by microsomes or a genuine property of major constituents of these fractions.

Table 3. *Distribution of ouabain-sensitive adenosine-triphosphatase activity in subcellular fractions from brain cortex*

Fractions were prepared from a 10 % (w/v) homogenate in 0.25M-sucrose at the centrifugal forces and times indicated. Each sediment, except the microsomal, was washed twice by resuspending in 0.25M-sucrose and centrifuging again at the same force and for the same time. Wash supernatants were combined with the initial supernatant before proceeding with the next stage of the fractionation. Final resuspensions were made in 0.25M-sucrose. Fractions 1 and 2 were prepared in the 6886 head of the MSE Major refrigerated centrifuge at 0°, the superspeed attachment of which was used for the preparation of fractions 3 and 4. The no. 40 head of the Spinco model L ultracentrifuge was used in preparing fractions 5 and 6. All fractions were examined under the phase-contrast microscope, and the observed components were as indicated. Samples of each fraction were incubated for 3 min. at 37° in a medium containing (final concentrations): imidazole-HCl buffer, pH 7.5 (10 mM), NaCl (120 mM), KCl (25 mM), MgCl₂ (1.25 mM) and ATP (disodium salt, buffered to pH 7.5 with tris) (1.25 mM). The reaction was stopped by the addition of ice-cold trichloroacetic acid to a final concentration of 7 %. After the precipitated protein had been spun down, orthophosphate in the supernatant was measured.

Fraction no.	Fraction separated	Components	Protein (mg./fraction)	Rate of orthophosphate liberation (μ moles/hr.)			
				Per fraction		Inhibited by ouabain	
				Control	With ouabain	Per fraction	Per mg. of protein
—	—	Whole homogenate	194	4045	2800	1245	6.4
1	At 800g for 10 min.	Nuclei + unbroken cells + large fragments	42	600	330	270	6.4
2	At 2 000g for 10 min.	Mitochondria + nuclei	14	165	120	45	3.2
3	At 10 000g for 10 min.	Mitochondria	23	580	410	170	7.4
4	At 16 000g for 10 min.	Mitochondria	18	245	190	55	3.1
5	At 20 000g for 10 min.	Mitochondria + microsomes	16	305	215	90	5.6
6	At 104 000g for 60 min.	Microsomes	31	810	310	500	16.1
	Supernatant	—	15	125	125	0	0
	Percentage recovery	—	80	70	61	90	—

*Respiration in reconstituted systems of
mitochondria and microsomes*

Although the mitochondrial fraction was partly sensitive to ouabain, both in its respiration and adenosine-triphosphatase activity, it was nevertheless decided to find out whether the ouabain-sensitive portion of the respiration could be increased by adding microsomes. It appears likely that the microsomal fraction, through its action as an adenosine triphosphatase, might be a pacemaker for part of the respiration in the whole homogenate, and in this way be responsible for at least part of the sensitivity of respiration to ouabain.

This experiment presupposes that the mitochondria are tightly coupled with and sensitive to respiratory control by ADP or orthophosphate resulting from extramitochondrial adenosine triphosphatases. The effect of hexokinase or ADP was therefore used as a control in each experiment; optimum amounts of each caused the same increase in respiration, i.e. 50–100 % of the unstimulated level (e.g. Table 4). The addition of microsomes to mitochondria stimulated respiration about 70 % (Fig. 7). Rates of oxygen consumption were linear whether microsomes were present from the start of the incubation or were added after 30 min. The stimulation of mitochondrial respiration by increasing amounts of microsomes (Table 4) reached a plateau 80 % greater than the unstimulated level, when the ratio of microsomal to mitochondrial protein was 4.5:1. (The relative amounts of microsomes to mitochondria in the whole homogenate would correspond to the addition of 0.6 mg. of microsomes in this experiment.) In the presence of ouabain the Q_{O_2} increased in a way almost parallel to that of the total on the addition of up to 4.5 mg. of microsomes, indicating that stimulation of respiration was almost insensitive to ouabain. When 7.5 mg. of microsomes was added the existing ouabain sensitivity was decreased. These results are puzzling in view of the fact that the adenosine-triphosphatase activity of the added microsomes was 60 % sensitive to ouabain. It is possible that the large increase in adenosine-triphosphatase activity caused by the addition of microsomes made this enzymic activity no longer the sole factor limiting the concentration of ADP or orthophosphate, and hence the respiration. The substrate (ATP) concentration may also be a factor limiting the rate of ADP and orthophosphate generation when the adenosine-triphosphatase activity is high. To test this possibility hexokinase was added to respiring mitochondria to increase the total concentration of ADP-generating enzymes. The Q_{O_2} was raised from 35 to 48 (Table 4). The addition of ouabain decreased the Q_{O_2} to 26 in the absence of hexokinase, but was without effect in its

presence. On the other hand, the Q_{O_2} , when stimulated to 54 by adding microsomes, was still subject to inhibition by ouabain.

The results show that, when respiration is sufficiently increased by an adenosine triphosphatase that is insensitive to ouabain (hexokinase), the ouabain-sensitive component of respiration is

Table 4. *Stimulation of mitochondrial respiration by microsomes, hexokinase and adenosine diphosphate, and the effects of ouabain*

Portions of mitochondrial suspension (1.2–3.2 mg. of protein) in medium A were incubated in medium B (see the text) with the additions indicated, and the O_2 consumption was measured over 60 min. at 37°. Amounts of microsomes were measured as mg. of protein. Each experiment was typical of three such similar experiments.

	Additions	Q_{O_2} (μ l. of O_2 /mg. of mitochondrial protein/hr.)	
		Control	With 50 μ M-ouabain
Expt. 1	None	62	47
	Microsomes (1.5 mg.)	93	73
	Microsomes (4.5 mg.)	112	100
	Microsomes (7.5 mg.)	112	106
Expt. 2	None	35	26
	Microsomes (3.0 mg.)	54	41
	Hexokinase (2 μ g.)	48	48
Expt. 3	None	41	—
	Hexokinase (2 μ g.)	57	—
	ADP (2 mM)	61	—

Fig. 7. Stimulation of respiration by microsomes of a mitochondrial fraction of brain cortex. Portions (2.45 mg. of protein) of mitochondrial suspension in medium A were incubated in medium B (see the text) and the O_2 uptake was measured over 60 min. at 37°. ●, Control; ○, with microsomes (3.0 mg. of protein) added to the mitochondria before incubation; △, mitochondria incubated without microsomes for first 30 min. and microsomes (3.0 mg. of protein) added from the side arm at 30 min.

abolished. If, on the other hand, the respiration is increased by the addition of adenosine triphosphatase that is partly ouabain-sensitive, then in spite of the increase in respiration the ouabain-sensitive part of the respiration still persists. It follows from these observations that both components of the microsomal adenosine triphosphatase can act as regulators of mitochondrial respiration.

DISCUSSION

Parallelism of adenosine-triphosphatase activity and respiration in brain homogenates

Effects of Na⁺ ions, K⁺ ions and ouabain. The adenosine-triphosphatase activity of rabbit-brain homogenate described above shows characteristics similar to those of the adenosine triphosphatase found in peripheral crab nerve (Skou, 1957, 1960), in isolated brain microsomes (Deul & McIlwain, 1961; Aldridge, 1962; Skou, 1962), and in rat-brain homogenate (Hess, 1962). The main finding of the present work is that respiration and adenosine-triphosphatase activity of the homogenate show several similarities. Both exhibit an ouabain-sensitive component identical in magnitude with the activation caused by Na⁺ and K⁺ ions. Further, there is a quantitative correlation between respiration and adenosine-triphosphatase activity (Fig. 3), inasmuch as inhibition of each by submaximal concentrations of ouabain leads to identical relative decreases: any change in one process was accompanied by the same relative effect in the other.

Another such correlation is the similarity in the Na⁺ ion requirement of about 100 mM for maximal rates of both processes (Figs. 1 and 2). However, K⁺ ions activate the adenosine-triphosphatase activity at much lower concentrations, for 5 mM-K⁺ ion is sufficient to elicit 80 % of the activity of the Na⁺ ion-plus-K⁺ ion-activated adenosine triphosphatase. Of necessity, the incubation mixture used in the measurement of respiration never contained less than about 6 mM-K⁺ ion which was derived from the initial tissue. It is not possible, therefore, to compare the respiration and adenosine-triphosphatase activity at K⁺ ion concentrations below 6 mM. The high respiration in the absence of added K⁺ ions is compatible with almost maximum activation of the adenosine-triphosphatase activity by the endogenous K⁺ ions.

The finding that both increases (due to a synergic action of Na⁺ and K⁺ ions) and decreases (due to ouabain) of the adenosine-triphosphatase activity are accompanied by parallel changes in the rate of respiration is suggestive of a causal relationship. This relationship explains the observation of the stimulation of the respiration of brain homogenates by Na⁺ ions and the inhibition by ouabain (Whit-

tam, 1962*a*); it also provides an explanation for the finding that the stimulation of respiration of brain slices either by electrical pulses (Gore & McIlwain, 1952) or by K⁺ ions (Dickens & Greville, 1935; Hertz & Schou, 1962) is dependent on the presence of Na⁺ ions in the medium.

Effects of Ca²⁺ ions and ethylenediaminetetra-acetic acid. ATP hydrolysis in the presence of optimum amounts of Na⁺ and K⁺ ions was inhibited by Ca²⁺ ions, as well as by ouabain, in agreement with the findings of Gore (1951) and Hess (1962). A comparison of the effects of Ca²⁺ ions in the presence and absence of ouabain gives some indication of their locus of action (Fig. 5). Although the total adenosine-triphosphatase activity was inhibited by Ca²⁺ ions, it appears that the ouabain-sensitive component was more easily inhibited by Ca²⁺ ions than the ouabain-insensitive component. The response of the respiration to Ca²⁺ ions is similar to that of the adenosine-triphosphatase activity, in that the ouabain-sensitive component is more sensitive to Ca²⁺ ions than is the ouabain-insensitive component. Previous studies have shown inhibition of the respiration of brain homogenates by Ca²⁺ ions (Elliott & Libet, 1942), but the cause of the effect was not elucidated. It is clear from the present results that one cause of this inhibition is the decrease in the Na⁺ ion-plus-K⁺ ion-activated adenosine-triphosphatase activity. This may also account for Krebs's (1950) observation that the respiration of brain slices is depressed by Ca²⁺ ions.

Ca²⁺ and K⁺ ions have antagonistic effects on the respiration of brain slices (Dickens & Greville, 1935; Canzanelli, Rogers & Rapport, 1942) and on their phosphocreatine content (McIlwain, 1952). The present results suggest that both effects may be due to the preferential inhibition by Ca²⁺ ions of the Na⁺ ion-plus-K⁺ ion-stimulated adenosine triphosphatase.

An anomalous result in the general parallelism that has been found between the respiration and adenosine-triphosphatase activity is the disparity in the concentrations of Ca²⁺ ions required to give comparable inhibition of the two processes (Figs. 4 and 5). This anomaly may be related to the fact that much higher concentrations of tissue protein (about 100 times) were present during measurements of respiration than of adenosine-triphosphatase activity. This difference in protein concentration may affect the concentration of free Ca²⁺ ions, for Ca²⁺ ions in the cell exist largely in a bound form. For example, in the squid giant axon it has been calculated that only 1 % of the endogenous Ca²⁺ ions is present as free ions (Hodgkin & Keynes, 1957). It is likely, therefore, that in the measurement of respiration the final concentration of free Ca²⁺ ions was very much less than the amount added in the medium.

The effects of EDTA on adenosine-triphosphatase activity may be compared with those of Ca^{2+} ions, on account of the chelating effect of this compound on Ca^{2+} ions. The stimulation of adenosine-triphosphatase activity by EDTA is compatible with the removal of the inhibitory effect of Ca^{2+} ions since both EDTA and Ca^{2+} ions affect particularly the Na^+ ion-activated component (Fig. 6). If EDTA exerts its effect by chelating endogenous Ca^{2+} ions, it is evident that the endogenous Ca^{2+} ions are sufficient to inhibit partially the Na^+ ion-plus- K^+ ion-activated adenosine triphosphatase and that the addition of further Ca^{2+} ions causes greater inhibition.

Respiration was little affected by EDTA in concentrations up to 25-fold greater than those needed to elicit maximum stimulation of the adenosine triphosphatase. This finding contrasts with the parallel inhibition by Ca^{2+} ions of both respiration and adenosine-triphosphatase activity. No simple explanation can be offered to account for this lack of correlation.

Control of respiration

With the exception of the results with EDTA, the parallel effects of Na^+ ions, K^+ ions, Ca^{2+} ions and ouabain on both the respiration and adenosine-triphosphatase activity, and the results of experiments on the reconstituted system of mitochondria and microsomes, can best be explained in terms of the Na^+ ion-plus- K^+ ion-activated adenosine triphosphatase acting as a pacemaker for part of the respiration. A feedback control of respiration is envisaged: in a tightly coupled system the availability of ADP or orthophosphate for oxidative phosphorylation limits the rate of oxygen uptake (see Chance & Williams, 1956). The results suggest that changes in the activity of the Na^+ ion-plus- K^+ ion-activated adenosine triphosphatase evoke corresponding changes in the concentrations of ADP and orthophosphate, and can thereby govern the rate of respiration. This adenosine-triphosphatase activity appears to be closely associated with the ion-transport process occurring at the membrane, and in human erythrocytes is manifest only when Na^+ and K^+ ions are being actively transported. Active cation transport can therefore be regarded as a membrane adenosine triphosphatase. It has already been proposed that, in slices of brain and kidney cortex, active cation transport acts as a pacemaker for respiration, since changes in the active uptake of K^+ ions are accompanied by parallel changes in Q_{O_2} (Whittam, 1961, 1962a; Whittam & Willis, 1963). Liver slices behave in a similar way (Elshove & van Rossum, 1963). The present results, and those with kidney-cortex homogenate (Blond & Whittam, 1964), show that the link between energy utilization by active cation

transport and energy production is the Na^+ ion-plus- K^+ ion-activated adenosine triphosphatase, and provide some answer to the questions of Keynes (1957) and Quastel (1961) on the influence of active transport on cell metabolism.

Subcellular fractions

It seems likely that the effect of ouabain on the brain mitochondrial fraction was the result of contamination by microsomes or other particles. It is well known that the usual preparations of brain mitochondria lack homogeneity (see Whittaker, 1963), and electron microscopy reveals considerable contamination by vesicles and membranes (Petrushka & Giuditta, 1959).

Support for this interpretation is provided by the work of Jöbsis (1963), who described the effect of ouabain on the adenosine-triphosphatase activity of two components of a mitochondrial fraction from brain. The activity of one component was stimulated by Na^+ and K^+ ions and inhibited by ouabain, whereas that of the other component was little affected by these agents. He showed that the sensitivity of the mitochondrial preparation to Na^+ and K^+ ions and to ouabain was caused by contamination of the mitochondria by other particles. A comparison of our preparative procedure with that of Jöbsis (1963) shows that both the ouabain-sensitive and -insensitive fractions that he described would be present in the mitochondrial preparation used in our experiments.

Ouabain sensitivity of cell fractions. Separation of the homogenate into fractions shows that ouabain-sensitive adenosine-triphosphate activity is concentrated in the microsomal fraction. These results are consistent with those of Deul & McIlwain (1961) and of Bonting, Caravaggio & Hawkins (1962), who showed a similar distribution of the Na^+ ion-plus- K^+ ion-activated adenosine triphosphatase in brain fractions. Comparison of these results provides further evidence that ouabain acts on the same extramitochondrial process as do Na^+ and K^+ ions. Work on more well-defined nervous tissue, such as neurones (Cummins & Hyden, 1962) and peripheral nerve (Baker, 1963), unequivocally implicates the cell membrane as the location of the Na^+ ion-plus- K^+ ion-activated adenosine-triphosphatase activity. It is likely, therefore, that the high proportion of this enzymic activity in brain microsomal fractions is due to a large proportion of the fragmented membrane, a conclusion supported by morphological evidence showing that the microsomes from brain contain membranous material (Hanson & Toschi, 1959) and by the association of this activity with membrane structures rather than ribonucleic acid granules in the microsomal fraction (Schwartz, Batchelard & McIlwain, 1962). It is reasonable to suppose that

the cell membrane is the only site of action of ouabain, and that the distribution of ouabain-sensitive activity in the subcellular fractions arises from a similar distribution of membrane fragments.

SUMMARY

1. A study has been made of the dependence of the respiration on the adenosine-triphosphatase activity of brain-cortex homogenates. The effects of factors (Na^+ ions, K^+ ions, Ca^{2+} ions and ouabain) that are known to influence active movements of ions in the intact tissue were investigated.

2. When the concentrations of Na^+ and K^+ ions in the medium were varied interchangeably, adenosine-triphosphatase activity was maximal with 30 mM- K^+ ion plus 120 mM- Na^+ ion; activity was least with 150 mM- K^+ ion plus 2.5 mM- Na^+ ion or with 152.5 mM- Na^+ ion. In the presence of ouabain activity was unaltered by changes in the concentrations of Na^+ and K^+ ions and stayed constant at the minimum value that was found in the absence of K^+ ions or on replacement of Na^+ ions with choline.

3. Oxygen consumption by brain homogenates was maximal with high Na^+ ion and low K^+ ion concentrations, and decreased as the concentrations of these ions were interchanged until with 150 mM- K^+ ion it had fallen to a minimum value that was not further decreased by ouabain. Respiration in the presence of ouabain was unaffected by changes in the concentrations of Na^+ and K^+ ions and stayed constant at the Q_{O_2} found on replacement of Na^+ ions with K^+ ions or choline.

4. Concentrations of ouabain giving submaximal inhibition caused parallel decreases in the oxygen consumption and adenosine-triphosphatase activity.

5. Ca^{2+} ions decreased the rates of both respiration and ATP hydrolysis, the latter being inhibited by much lower concentrations than was respiration. Ouabain diminished the magnitude of the inhibition by Ca^{2+} ions.

6. Adenosine-triphosphatase activity was stimulated by low concentrations of EDTA and inhibited by higher concentrations. In the presence of ouabain or absence of Na^+ ions, stimulation was prevented and only the decrease was observed. Respiration was little affected by EDTA at concentrations up to 5 mM.

7. The distribution of ouabain-sensitive adenosine-triphosphatase activity in fractions of brain cortex showed that the specific activity in the microsomal fraction was nearly three times as great as that in the other fractions, except the supernatant, which had zero activity.

8. The addition of microsomes to respiring mitochondria stimulated oxygen consumption in a way suggesting that both the ouabain-sensitive and

-insensitive components of the microsomal adenosine-triphosphatase activity control the respiration.

9. It is concluded that the Na^+ -ion-plus- K^+ -ion-activated adenosine triphosphatase, which is a part of the active cation transport system, is a pace-maker for 30–40 % of the respiration in brain cortex.

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The Regulation of Kidney Respiration by Sodium and Potassium Ions

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In biochemical studies on cellular energetics more attention has been devoted to energy production during intermediary metabolism than to energy utilization. Processes that utilize energy have not, as a rule, been investigated with a view to determining their role in the energy metabolism of the cell. Yet, when considering the cell as an integrated unit, it is evident that there must be interplay between energy production and utilization. The interrelationship between active cation transport and respiration is particularly suitable for the study of such a coupling, since active transport is continuous in the intact cell and does not require nervous or hormonal stimulation. Brain and kidney cortex, for example, have high rates of respiration and active transport, and the latter is a major energy-utilizing reaction in both tissues. In slices of these tissues evidence has been obtained that ion transport acts as a pacemaker of respiration, largely because decreases in the rate of uptake of K^+ ions, induced by means that were believed not to inhibit respiration directly, are nevertheless accompanied by parallel decreases in respiration (Whittam, 1961, 1962; Whittam & Willis, 1963). There is thus an interdependence of the rates of respiration and active movements of cations. Another parallelism is that between adenosine-triphosphatase activity and active cation transport (Skou, 1957; Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961; Tosteson, Moulton & Blaustein, 1960; Wheeler & Whittam, 1962).

Thus on the one hand ion transport and respiration in slices are connected, and on the other hand there is a linkage between ion transport and adenosine-triphosphatase activity. The present experiments were designed to see whether this

adenosine triphosphatase acts as a regulator of respiration, thus providing the mechanism by which active ion transport regulates respiration. Factors known to influence the rate of ion transport have therefore been investigated for their effects on the rates of respiration and of ATP hydrolysis in homogenates. Further, to see whether these factors affect respiration directly, their influence on the respiration of isolated mitochondria has been examined.

The results show that the respiration of rabbit-kidney homogenate is markedly affected by the concentrations of Na^+ ions, K^+ ions, Ca^{2+} ions and ouabain in the medium. A control of respiration has been demonstrated by an adenosine triphosphatase sensitive to these ions which is believed to be involved in the active transport of Na^+ and K^+ ions.

METHODS

Preparation of tissue suspensions. Adult rabbits of both sexes were stunned by a blow on the neck and bled from the throat. The kidneys were excised and, after removal of the capsule, each was bisected longitudinally and the exposed medulla cut away. The cortex (about 4 g.) was disrupted in about 12 ml. of ice-cold medium containing sucrose (0.25 M) and imidazole-HCl buffer, pH 7.8 (10 mM), in a Potter-Elvehjem-type homogenizer with a smooth glass tube and Teflon pestle (total clearance 0.01 in.). The pestle, revolving at 700 rev./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 0.25 M-sucrose medium.

Preparation of kidney mitochondria. A 10% (w/v) homogenate was centrifuged at 850g for 10 min. at 0° (MSE Major refrigerated centrifuge, 6886 head; 2300–2400 rev./