

The Effect of Chemical Agents on the Turnover of the Bound Phosphate Associated with the Sodium-and-Potassium Ion-Stimulated Adenosine Triphosphatase in Ox Brain Microsomes

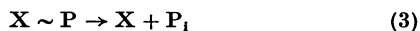
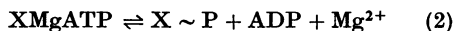
By R. RODNIGHT

*Department of Biochemistry, Institute of Psychiatry
(British Postgraduate Medical Federation, University of London),
De Crespigny Park, London S.E.5, U.K.*

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1. The effect of chemical agents on the turnover of the Na⁺-dependent bound phosphate and the simultaneous Na⁺-dependent hydrolysis of ATP by a membrane preparation from ox brain was studied at an ATP/protein ratio of 12.5 pmol/μg. 2. The agents were added immediately after phosphorylation of the preparation in a medium containing 50 mM-sodium chloride and 2.5 μM-[γ-³²P]ATP. 3. Concentrations of sodium chloride above 150 mM, calcium chloride to 20 mM and suramin to 1.4 mM inhibited both phosphorylation and dephosphorylation and concomitantly slowed ATP hydrolysis. At 125 mM-sodium chloride dephosphorylation and hydrolysis were slightly slowed without affecting phosphorylation. 4. Ethanol to 1.6 M concentration inhibited dephosphorylation without affecting phosphorylation; the bound phosphate was increased and ATP hydrolysis slowed. 5. Ouabain to 4 mM concentration partially inhibited ATP hydrolysis and caused a transient (1-2 s) rise in bound phosphate followed by a rapid fall to a lower plateau value, which eventually declined to zero by the time ATP hydrolysis was complete. 6. Of the detergents examined Lubrol W, Triton X-100 and sodium deoxycholate had no significant effect on turnover. Sodium dodecyl sulphate and sodium decyl sulphate to 3.5 mM and 20 mM respectively completely inhibited turnover and ATP hydrolysis and stabilized the bound phosphate.

It is well established that the hydrolysis of ATP by the Na⁺,K⁺,Mg²⁺-ATPase* enzyme system of cell membrane fragments is closely associated with a Na⁺-dependent formation of phosphate bound to a protein component of the membrane (Rodnight, Hems & Lavin, 1966; Fahn, Koval & Albers, 1966a, 1968; Nagano *et al.* 1967; Kanazawa, Saito & Tonomura, 1967; Schoner, Beusch & Kramer, 1968; Skou & Hilberg, 1969). The simplest explanation of this observation postulates that the phosphorylated component represents a covalent intermediate in ATPase action according to the following sequence:



where X is either the enzyme itself or a membrane protein acting as a phosphate acceptor for a coupled kinase-phosphatase system, and where Na⁺ stimulates reaction (2) and K⁺ reaction (3). Consistent

* Abbreviation: Na⁺,K⁺,Mg²⁺-ATPase, Na⁺ + K⁺ + Mg²⁺-stimulated adenosine triphosphatase.

with this formulation is the finding that at low concentrations of ATP the steady-state plateau concentration of Na⁺-dependent bound phosphate is decreased by K⁺, apparently by increasing the turnover rate and thus under conditions of limited substrate diminishing the number of sites reacting. The additional Mg²⁺-dependent transphosphorylation step proposed by Fahn *et al.* (1968) can be accommodated in the general mechanism without affecting the role of the univalent cations. On the whole, the evidence supporting the existence of an obligatory phosphorylated intermediate in ATP hydrolysis by this system is very strong, although in my view it is preferable to avoid the term 'phosphorylated intermediate' until more is known of the structure and nature of the system.

It is a feature of the kinetics of the formation of Na⁺-dependent bound phosphate in cerebral membrane fragments that even in a K⁺-free medium the steady-state rate declines to zero as ATP hydrolysis (by enzyme action requiring only added Na⁺) approaches completion. In the undenatured state of the membrane, therefore, the bound phosphate

is labile. After acid denaturation of the membrane protein the bound phosphate exists in a relatively stable acyl linkage, probably to the γ -carboxyl group of a glutamic acid residue (Kahlenberg, Galsworthy & Hokin, 1967). An apparently stable form of Na^+ -dependent bound phosphate can also be detected at neutral pH after denaturation with sodium dodecyl sulphate (Nagano *et al.* 1967), but under these conditions less is known of the nature of the bond. In the work reported here, factors influencing bound phosphate stability at neutral pH were investigated. The present paper describes attempts to stabilize the bond by the addition of agents, mainly inhibitors of ATPase action, to the fully phosphorylated system. The following paper (Goldfarb & Rodnight, 1970) reports experiments suggesting that the instability is related to the presence in the membrane preparations of traces of tightly bound K^+ and attempts to stabilize the bound phosphate by removal of this bound K^+ .

MATERIALS

Reagent-grade chemicals and deionized glass-distilled water were used throughout. The various detergents used were obtained from the following sources: sodium dodecyl sulphate from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., sodium decyl sulphate from Mann Research Laboratories, New York, N.Y., U.S.A., sodium deoxycholate and Triton X-100 from BDH Chemicals Ltd., Poole, Dorset, U.K., Lubrol W from Imperial Chemical Industries Ltd., London W.C.1, U.K. Ouabain (G-strophanthin) was obtained from BDH Chemicals Ltd. and suramin (Antrypol) from Imperial Chemical Industries Ltd., Wilmslow, Ches., U.K.

METHODS

Preparation of membrane fraction. The procedure used was a modification of that described for ox brain by Rodnight *et al.* (1966). The following steps were carried out at the slaughter-house within 15 min of the death of the animal. The brain was freed of meninges and divided sagittally. Each hemisphere was separated from the underlying basal structures, sealed in a polyethylene bag and brought to the laboratory in ice, where it was immediately stored at -20°C for a maximum of 4 weeks. (In some later work the brain was frozen at the slaughter-house by immersion of the sealed bag in methanol cooled to -80°C with solid CO_2 and then brought to the laboratory in powdered solid CO_2 .)

Dissection of grey matter was carried out at room temperature by scraping the surface of the hemisphere as it thawed with a spoon-shaped spatula of stainless steel. When the grey matter had thawed to a soft consistency it was discarded. As they were obtained, the fragments were transferred to a known weight of ice-cold 0.32M-sucrose in 0.5mM-tris-EDTA, pH 7, until the tissue concentration was 20% (w/v). After homogenization by the procedure of Rodnight *et al.* (1966), nuclei, mitochondria and unbroken cells were separated by a single spin at an integrated force of $1.25 \times 10^5 \text{g}\cdot\text{min}$ in the no. 21 rotor of

the Spincro model L centrifuge. The pellet was washed twice with sucrose solution by resuspension and centrifugation at the same force. Special care was needed in decanting the supernatant from the last spin as the pellet tended to be loosely packed at this stage. The supernatants were combined and centrifuged for $5 \times 10^6 \text{g}\cdot\text{min}$ to deposit the crude membrane fraction. The latter, resuspended in 0.32M-sucrose, was essentially the product used by Rodnight *et al.* (1966). In the present work the membrane fragments at this stage were suspended in 1M-sucrose in 0.5mM-tris-EDTA, pH 7, and the dispersion was centrifuged for $9.6 \times 10^6 \text{g}\cdot\text{min}$ in the SW 25 or no. 30 rotor of the Spincro model L centrifuge. The procedure yielded a thin white pellicle on the surface of the sucrose solution, a tightly packed pellet at the bottom of the tube covered with a layer of loosely packed material and particles that remained suspended in the bulk of the sucrose solution. These particles, together with the loosely packed fraction of the pellet, contained about 60% of the total protein and 80% of the $\text{Na}^+, \text{K}^+, \text{Mg}^{2+}$ -ATPase activity. The two fractions were collected by pipette, combined and diluted with 0.5mM-tris-EDTA, pH 7, to a sucrose concentration of 0.3M, and the particulate material was spun down. Finally the pellets were washed twice in the centrifuge ($5 \times 10^6 \text{g}\cdot\text{min}$) with 4mM-imidazole-HCl buffer, pH 7.4. The preparation was stored in small test tubes at -20°C suspended in the same buffer at a protein concentration of 5 or more mg/ml.

The binding and ATPase activities were well maintained under these conditions, but it was noted that preparations stored at a concentration of less than 5mg of protein/ml tended to deteriorate. Immediately before an experiment a sample of the preparation was thawed, diluted to a protein concentration of 2mg/ml with 4mM-imidazole-HCl, pH 7.4, buffer and briefly dispersed in a test-tube homogenizer. Resuspension was necessary because, in contrast with membrane material stored in sucrose solutions, the preparation tended to form a coacervate on freezing.

Measurement of enzyme activities. The conditions chosen were optimum for observing the rate of decline of bound phosphate as ATP hydrolysis approached completion. In general these were those of Rodnight *et al.* (1966), except that the ATP concentration was $2.5 \mu\text{M}$ and the membrane protein concentration $200 \mu\text{g}/\text{ml}$; total radioactivities added as $[\text{}^{32}\text{P}]\text{ATP}$ were in the range 10^5 – $5 \times 10^5 \text{c.p.m.}$ The basal medium contained (final concentrations) 50 mM-NaCl, 30mM-tris-HCl buffer, pH 7.4 and $2.5 \mu\text{M}$ - MgCl_2 ; the final volume was 2.5ml. The reaction was started by the addition of membrane protein (0.25ml) and stopped by the addition of an equal volume of ice-cold 0.93M-trichloroacetic acid. The reaction mixture was magnetically stirred as described by Rodnight *et al.* (1966).

Bound radioactivity in the acid-washed protein precipitates was determined either on planchets in an end-window counter after dissolution of the protein in 98% (w/v) formic acid or by Čerenkov radiation in a liquid-scintillation spectrometer after solution in 6ml of 0.1M-NaOH. ^{32}P radioactivity extracted as phosphomolybdic acid by organic solvent in the ATPase assay method was also determined by one of these two methods; in the Čerenkov-radiation method a small sample (usually 0.25ml) of the organic layer was diluted with 6ml of 95% (v/v) ethanol. The efficiency of counting ^{32}P by the Čerenkov-radiation method was approx. 40%.

[γ -³²P]ATP was prepared as described by Rodnight *et al.* (1966). Protein was determined by the method of Miller (1959).

RESULTS

Enzyme activities of membrane preparation

Compared with the crude preparation obtained by Rodnight *et al.* (1966) phosphate-binding activity was greatly enhanced: maximum values with 20 μ M-ATP were in the range 120–200 pmol of phosphate/mg of protein compared with 20–40 pmol of phosphate/mg of protein for the previous preparation. The total Na⁺,K⁺,Mg²⁺-ATPase activity was higher by some 75%; stimulation by Na⁺ was three- to four-fold. Phosphate binding also exhibited a greater sensitivity to Na⁺, maximum values sometimes being attained with 5–10 mM-sodium chloride; with the previous preparation 100 mM-sodium chloride was required. Not all preparations, however, were as sensitive as this and in determining Na⁺-dependent bound phosphate a concentration of 50 mM-sodium chloride was used as a routine to ensure maximum phosphorylation. Full discharge of the bound phosphate required 0.1 mM-potassium chloride with 5 mM-sodium chloride and 2 mM-potassium chloride with 100 mM-sodium chloride. ATP hydrolysis with 20 μ M-ATP was approximately doubled by the addition of 2 mM-potassium chloride

to a medium containing 100 mM-sodium chloride; in the crude preparation a K⁺-sensitive hydrolysis of 20 μ M-ATP could not be demonstrated. However, stimulation of the ATPase reaction by Na⁺ alone at ATP concentrations of 20 μ M or less was again observed, although it was less than in the crude material. Both the phosphate-binding and ATPase reactions were also only slightly increased by addition of Mg²⁺ (equimolar with ATP) to the medium, although EDTA was inhibitory. This was not unexpected since the preparation contains 20 nmol of bound Mg²⁺/mg of protein (Goldfarb & Rodnight, 1970).

The higher enzyme activities and greater sensitivity to cations of the present subfractionated and washed preparation appear to be due partly to removal of non-active protein and partly to its lower content of intrinsic K⁺. The role of bound cations in the binding and ATPase reactions at low ATP concentrations is the subject of the following study (Goldfarb & Rodnight, 1970). Here it may be noted that, compared with the crude material obtained by Rodnight *et al.* (1966), the K⁺ content of the present preparation was decreased from 120 to 27 \pm 3 nmol of K⁺/mg of protein; however, the Na⁺ content, at 90 nmol/mg of protein, was only slightly less than before.

The time-courses of the Na⁺-dependent phosphate

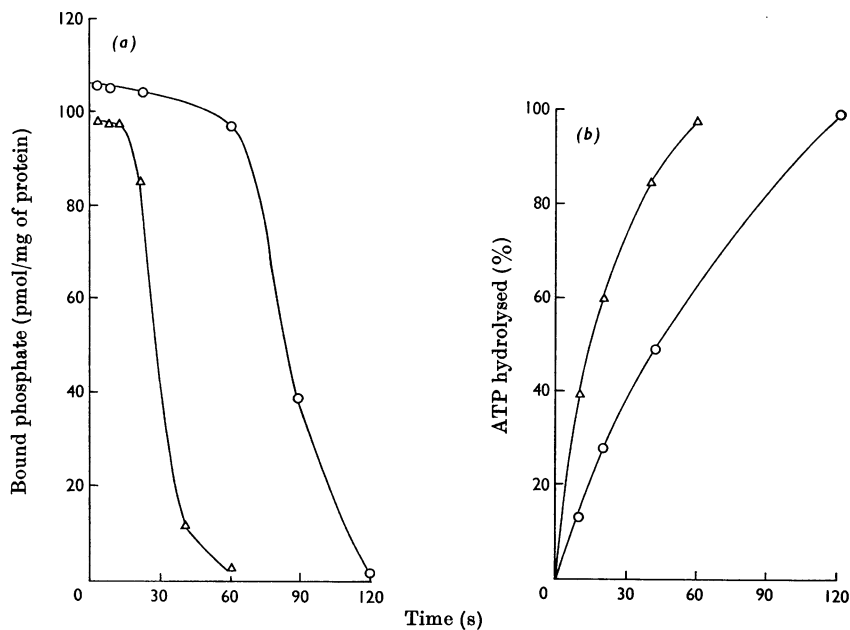


Fig. 1. Time-course at 37°C of (a) the formation and decline of bound phosphate and of (b) ATP hydrolysis by the microsomal preparation at two concentrations of ATP. \circ , 20 μ M-ATP; Δ , 2.5 μ M-ATP. The medium contained 50 mM-NaCl and 200 μ g of microsomal protein/ml. Other experimental details are given in the Methods section.

binding and ATPase reactions at ATP concentrations of 2.5 and 20 μM and a protein concentration of 200 $\mu\text{g}/\text{ml}$ are shown in Fig. 1. In both cases the plateau value of bound phosphate began to decline

after about 60% of the ATP had been hydrolysed. In the following paper (Goldfarb & Rodnight, 1970) evidence is presented supporting the conclusion that under these conditions of very low substrate

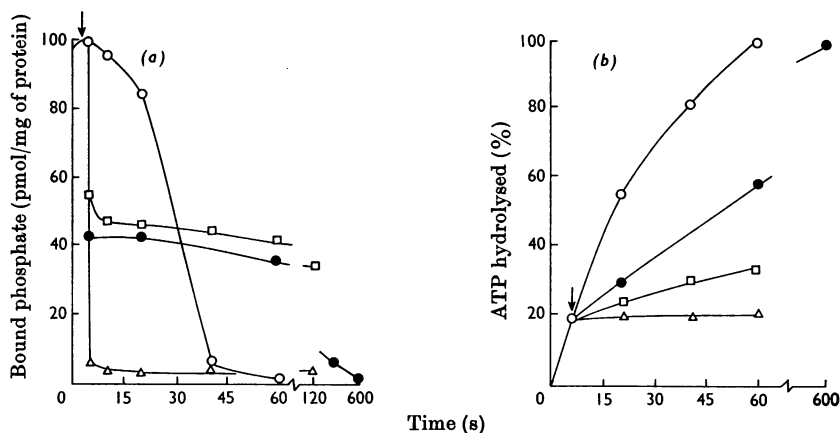


Fig. 2. Effect of a high concentration of Na^+ and Li^+ on the time-course at 37°C of (a) the formation and decline of bound phosphate and of (b) ATP hydrolysis by the microsomal preparation. The final concentrations of NaCl and LiCl were each 0.55 M. The additions were made at 4 s as indicated by the arrow. All media contained 50 mM- NaCl initially and 2.5 μM -ATP. For Na^+ two experiments with different preparations are shown (\square , \bullet) and for Li^+ one experiment (\triangle). The control time-course (\circ) is the mean of that observed in the three experiments. Other details are given in the legend to Fig. 1 and in the Methods section.

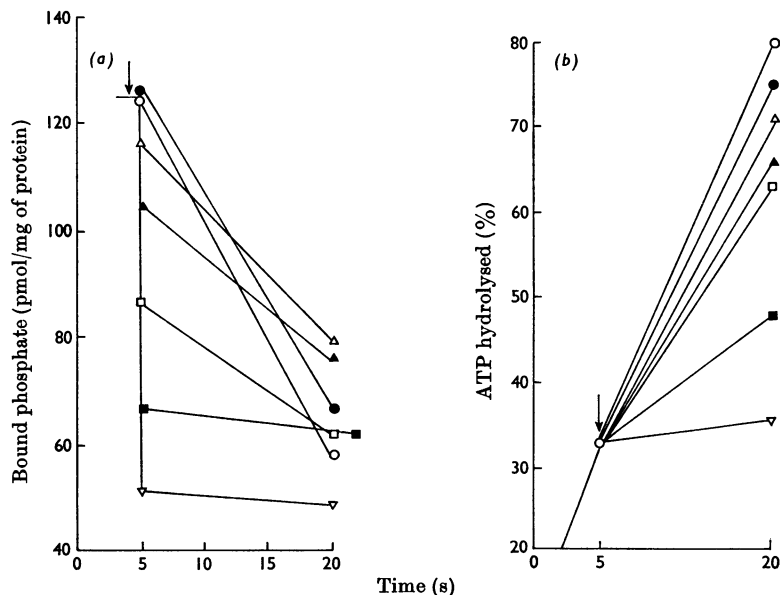


Fig. 3. Effect of high concentrations of NaCl on the time-course at 37°C of (a) the formation and decline of bound phosphate and of (b) ATP hydrolysis by the microsomal preparation. The NaCl solutions were added at 4 s as indicated by the arrow. All media contained 50 mM- NaCl initially and 2.5 μM -ATP. The final Na^+ concentrations were: \bullet , 125 mM; \triangle , 150 mM; \blacktriangle , 200 mM; \square , 250 mM; \blacksquare , 350 mM; ∇ , 550 mM. \circ , Control time-course.

concentration most, if not all, of the hydrolysis of ATP observed in a medium containing Na⁺ is due to the Na⁺,K⁺,Mg²⁺-ATPase stimulated by the bound K⁺ in the preparation.

Effects of agents on the stability of the Na⁺-dependent bound phosphate

The agents were added in a volume of 50–250 μl at 4 s after the reaction had been started. In control tubes the same volume of water or solvent was added instead of the agent. The reaction was stopped at a series of intervals starting at 5 s after zero time. Some difficulty was experienced in obtaining a full recovery of acid-denatured protein when the effects of detergents were being examined. In these experiments, therefore, the same volume of detergent was added to the control tubes after the reaction had been stopped with trichloroacetic acid. By centrifuging at 3000g for 20 min, 85–90% of the protein was recovered.

Univalent cations. There is ample evidence that under conditions of saturation kinetics Na⁺ competes with K⁺ for sites on the enzyme system (Skou, 1962; Ahmed, Judah & Scholefield, 1966). If Na⁺ is unable to replace K⁺ as an activator of the dephosphorylation step, the displacement of bound K⁺ with excess of Na⁺ should stabilize the bound phosphate. Flooding the phosphorylated system with Na⁺, however, had as its major effect an inhibition

of phosphorylation: thus a tenfold increase in Na⁺-concentration caused an immediate loss of about half the bound phosphate followed by the establishment of a new plateau that slowly declined after 1 min to zero in 10 min (Fig. 2). ATP hydrolysis continued after Na⁺ addition at a much diminished rate but was completed in 5–10 min. The inhibition of phosphorylation was first evident at 150 mM-sodium chloride (Fig. 3). At 125 mM concentration Na⁺ had no effect on the steady-state concentration of bound phosphate, but did slightly slow hydrolysis and therefore the rate of dephosphorylation; 100 mM-sodium chloride was without effect. There is thus a small range of Na⁺ concentrations near 125 mM at which the major effect of excess of Na⁺ is that of displacing K⁺ and slowing dephosphorylation; at higher concentrations this effect is obscured by the effect on phosphorylation. The effect was not due to Cl⁻, since sodium iodide had approximately the same effect; however, I have not excluded the possibility that both halide ions may be inhibitory. Opit, Potter & Charnock (1966) found equal ATPase rates with I⁻ and Cl⁻ as anion but found F⁻ to be inhibitory. In the present work a lower concentration of sodium fluoride (20 mM) slightly inhibited hydrolysis and dephosphorylation.

When Li⁺ instead of Na⁺ was added in excess, both phosphorylation and hydrolysis were completely inhibited (Fig. 2).

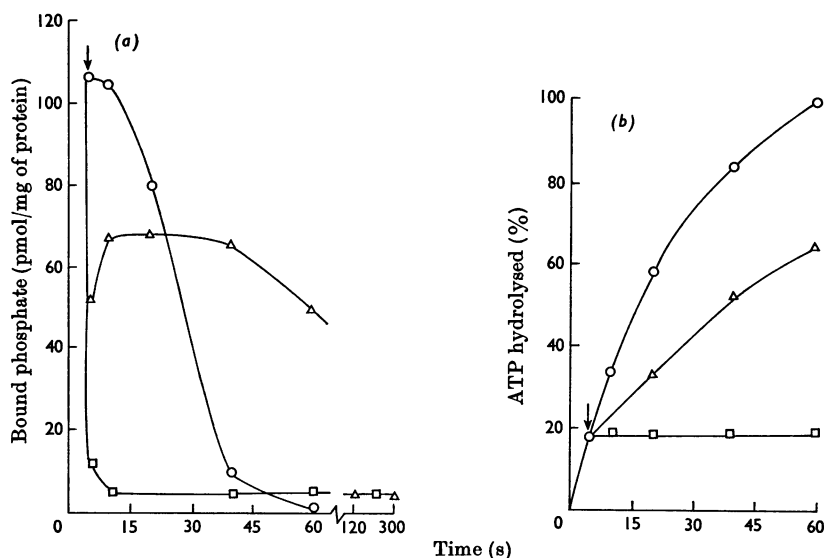


Fig. 4. Effect of CaCl₂ and EDTA on the time-course at 37°C of (a) the formation and decline of bound phosphate and of (b) ATP hydrolysis by the microsomal preparation. Additions to final concentrations of 20 mM were made at 4 s as indicated by the arrow. ○, Control (water); △, CaCl₂; □, EDTA. The medium contained 50 mM-NaCl and 2.5 μM-ATP. Other details are given in the legend to Fig. 1 and in the Methods section.

Bivalent cations. Ca^{2+} inhibits the $\text{Na}^+, \text{K}^+, \text{Mg}^{2+}$ -ATPase and the Na^+ -dependent formation of bound phosphate. When added to the fully phosphorylated system to a final concentration of 20mM, calcium chloride halved the concentration of bound phosphate within 1s, but over the next 5s it rose to about two-thirds of the control value and then slowly declined to near zero over a period of 2–3min (Fig. 4). ATP hydrolysis was 50% inhibited and was complete in 2min. This curious biphasic response in the first 5s after Ca^{2+} addition was reproducible and has not been found with any other agent used in the present study. It suggests that the inhibition may be occurring at more than one site on the system at different rates. Possibly Ca^{2+} first converts a proportion of the ATP into the calcium salt, but as Ca^{2+} becomes bound to the membrane components the concentration falls enough to allow MgATP to rephosphorylate a proportion of the sites. However, a similar biphasic response was found by Skou & Hilberg (1969) to K^+ added to a preparation phosphorylated by a high concentration of ATP.

It was argued by Rodnight *et al.* (1966) that, if Mg^{2+} as well as K^+ is required for the dephosphorylation step, chelating agents should have the effect of stabilizing the bound phosphate. In fact Rodnight *et al.* (1966) found that the addition of EDTA to the phosphorylated system caused a rapid loss of bound phosphate along with complete cessation of ATP hydrolysis and they tentatively interpreted

this as indicating that Mg^{2+} was required for protein phosphorylation but not for dephosphorylation. However, in this experiment bound phosphate was only measured 10s after EDTA addition, when the concentration was still 50% of the control value; partial stabilization therefore was not excluded. Reinvestigation of the effect over a series of time-intervals gave some evidence for stabilization at a very low concentration (Fig. 4). EDTA to 20mM concentration blocked ATP hydrolysis and decreased the concentration of bound phosphate from 96 to 12pmol/mg of protein within 5s of addition. This low concentration was stable for at least 3min at 37°C, during which interval ATPase activity remained completely inhibited. In the control series hydrolysis was complete in 1min, by which time the concentration of bound phosphate had fallen to 3.5pmol/mg of protein.

The addition of 1mM-magnesium chloride to the phosphorylated system stimulated ATP hydrolysis and accelerated the decline in the concentration of bound phosphate.

Suramin. This substance is a powerful inhibitor of ATPase action in membrane preparations (Schwartz, Bachelard & McIlwain, 1962). Added to 1.4mM concentration, suramin caused a very rapid loss of bound phosphate followed by a new plateau at about one-third of the control value; ATP hydrolysis was decreased to 10% of the control value (Fig. 5). The same result was obtained by

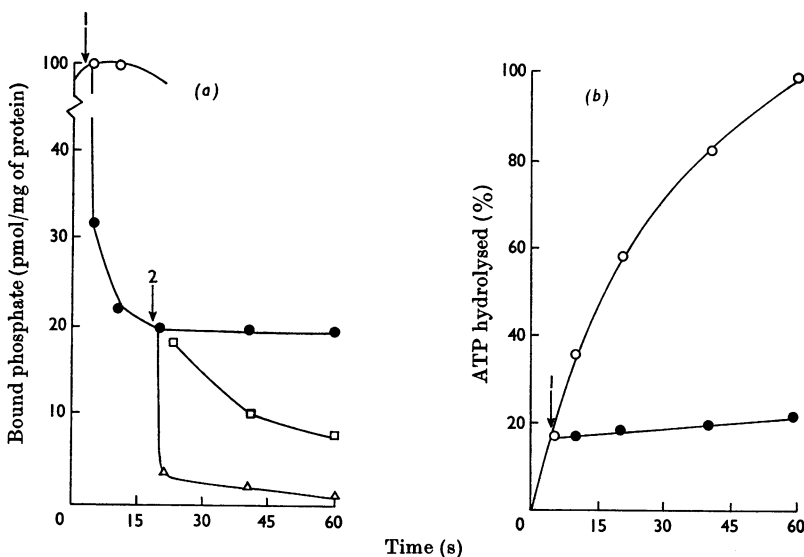


Fig. 5. Effect of suramin on the time-course at 37°C of (a) the formation and decline of bound phosphate and of (b) ATP hydrolysis by the microsomal preparation. Suramin to a final concentration of 1.4mM was added at 4s as indicated by arrow 1. \circ , Control (water); \bullet , suramin. At 19s (arrow 2) KCl (Δ) or EDTA (\square) was added to a concentration of 20mM. Only part of the control time-course is shown. The medium contained 50mM-NaCl and 2.5 μM -ATP. Other details are given in the legend to Fig. 1 and in the Methods section.

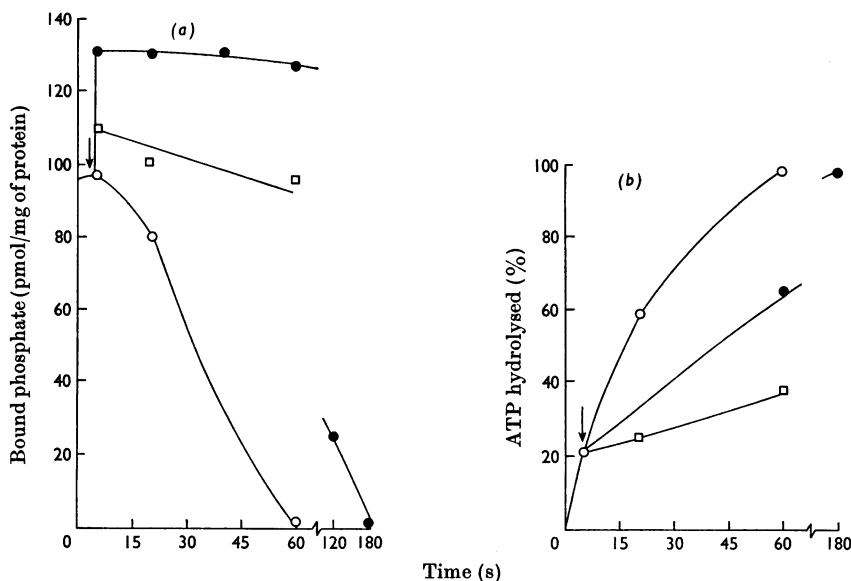


Fig. 6. Effect of ethanol on the time-course at 37°C of (a) the formation and decline of bound phosphate and of (b) ATP hydrolysis by the microsomal preparation. Ethanol to a final concentration of 10% (●) or 20% (□) was added at 4s as indicated by the arrow. In the control (○) water was added instead of ethanol. The medium contained 50mM-NaCl and 2.5 μM-ATP. Other details are given in the legend to Fig. 1 and in the Methods section.

incubation at 2°C instead of 37°C, the suramin being added at 2min instead of 4s: after the initial fall to 40pmol/mg of protein no further loss occurred over the next hour, although in this experiment also ATP hydrolysis continued at approx. 10% of the uninhibited rate. The low rate of ATP hydrolysis that continued after the addition of suramin suggested that the bound phosphate was still turning over. This was shown to be the case by adding excess of non-radioactive ATP, potassium chloride or EDTA to the system after suramin; in all cases a further decline in the concentration of bound phosphate occurred (Fig. 5).

Inhibition by suramin was reversible: over 90% of the original phosphate-binding and ATPase activities were recovered after the centrifugal washing of excess of suramin from the protein with imidazole buffer. When labelled protein similarly washed with buffer containing a high concentration (1.4mM) of suramin all the bound phosphate was lost, confirming the above conclusion that the suramin permitted a low rate of turnover.

Ethanol. Both cation transport and the Na⁺,K⁺,Mg²⁺-ATPase are inhibited by lower aliphatic alcohols (Israel, Kalant & LeBlanc, 1966). The effect of ethanol on the turnover of bound phosphate appears to be primarily on dephosphorylation (Fig. 6), although above 3M concentration some inhibition

of phosphorylation as well as dephosphorylation was evident. The selective action of the drug was confirmed by observing normal phosphorylation in a medium with 10% (1.6M) ethanol present from the start of the reaction: the concentrations of bound phosphate (pmol/mg of protein) at 2 and 5s respectively were 108 and 110 in the absence of ethanol and 110 and 126 with ethanol present. However, even at these high concentrations of ethanol some ATPase activity continued and the concentration of bound phosphate eventually declined to zero as hydrolysis approached completion (Fig. 6). Addition of potassium chloride to 10mM concentration 15s after addition of ethanol to 1.6M caused an immediate discharge of 50% of the bound phosphate to a new plateau value, but with no measurable increase in the partly inhibited ATPase reaction. The position here is therefore similar to that observed with suramin, where the residual uninhibited fraction of the enzyme system is associated with a plateau concentration of bound phosphate, which continues to turn over slowly. Acetone added to 5% (v/v) had the same action on the phosphorylated system.

Ouabain. Rodnight *et al.* (1966) found that ouabain increased the 10s concentration of Na⁺-dependent bound phosphate when the Na⁺ concentration was suboptimum (15mM-sodium chloride)

and decreased it when the Na^+ concentration was optimum (100mM-sodium chloride); the Na^+ -dependent ATPase activity was partly inhibited in both cases. In these experiments the microsomes were pretreated with ouabain for 5min before the reaction was carried out, and the stimulation at low Na^+ concentration was interpreted as indicating a displacement by the drug of intrinsic K^+ bound to the dephosphorylation site; the results also suggested that some inhibition of phosphorylation was occurring, particularly in the presence of 100mM-sodium chloride. To explore these findings further in the present work a high concentration (4mM) of ouabain was added to the fully phosphorylated system. To do this it was necessary to dissolve the ouabain in 20% (v/v) ethanol, giving a final concentration of ethanol in the reaction mixture of less than 1% (v/v); this was well below the concentration inhibiting dephosphorylation. Added in this way ouabain caused a transient rise in the concentration of bound phosphate followed within 1–2s by a rapid dephosphorylation to a new plateau concentration about 20% of the control value (Fig. 7). This low plateau value was maintained until ATP hydrolysis was nearly complete, when it declined to zero. This time-course, including the transient increase in the concentration of bound phosphate, was independent of the Na^+ concentration over the range 5–100mM; it was also observed when a much lower concentra-

tion of ouabain (0.1mM) was incorporated in the reaction mixture at the start of the reaction.

Oligomycin. Oligomycin is a weak inhibitor of the $\text{Na}^+, \text{K}^+, \text{Mg}^{2+}$ -ATPase (Van Groningen & Slater, 1963) and was found by Israel & Titus (1967) to increase the concentration and prolong the duration of the plateau value of Na^+ -dependent bound phosphate formed by ox brain microsomes. These results were confirmed in the present study and it was also noted that the concentration of bound phosphate in the presence of oligomycin eventually declined to zero in parallel with ATP hydrolysis (Fig. 8). Because of its low solubility it was not possible to add oligomycin to the system after phosphorylation.

Detergents. The action of detergents was studied since they could be expected to destroy or modify the structural integrity of the membrane fragments and so open the possibility of fractionating labelled proteins by chromatography or gel filtration. Of the detergents examined only the sodium alkyl sulphates were effective in stabilizing the bound phosphate: Triton X-100, Lubrol W and sodium deoxycholate had no effect, whereas sodium dodecyl sulphate added to a final concentration of 0.1% (3.5mM) completely stopped ATP hydrolysis and dephosphorylation (Fig. 9), as originally observed by Nagano *et al.* (1967). The stabilized bound phosphate, however, still showed some sensitivity to a

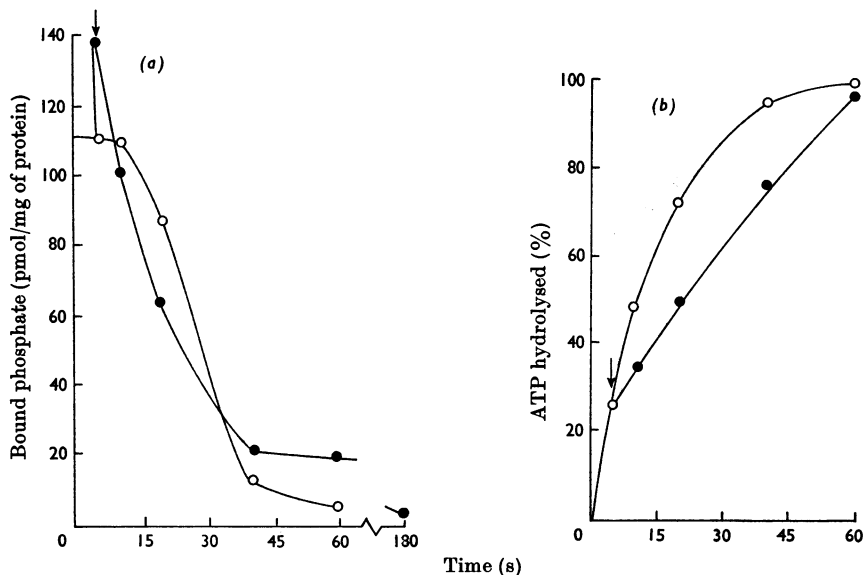


Fig. 7. Effect of ouabain on the time-course at 37°C of (a) the formation and decline of bound phosphate and of (b) ATP hydrolysis by the microsomal preparation. Ouabain (0.1mM in 20% ethanol) was added to a final concentration of 4mM at 4s as indicated by the arrow. The final ethanol concentration was less than 1%. ○, Control (water); ●, ouabain. The medium contained 50mM-NaCl and 2.5μM-ATP. Other details are given in the legend to Fig. 1 and in the Methods section.

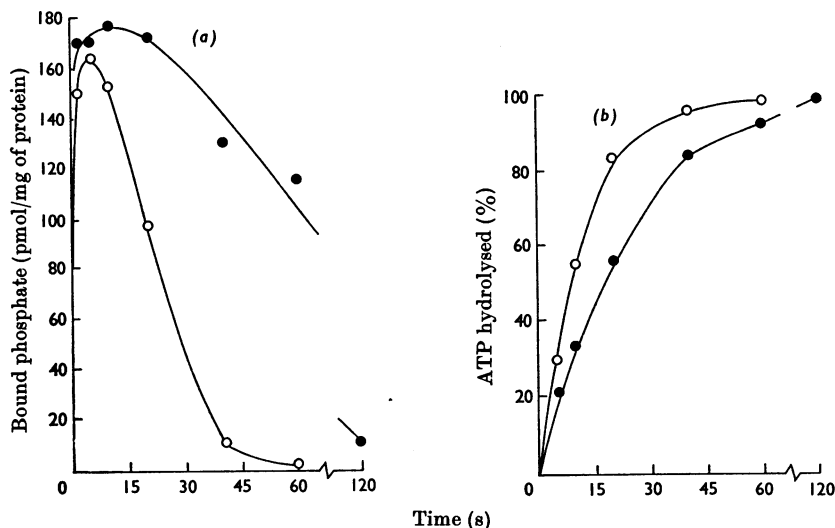


Fig. 8. Effect of oligomycin on the time-course at 37°C of (a) the formation and decline of bound phosphate and of (b) ATP hydrolysis by the microsomal preparation. In this experiment oligomycin (●) at 100 $\mu\text{g/ml}$ was present at the start. ○, Control. The medium contained 50 mM-NaCl and 2.5 μM -ATP. Other details are given in the legend to Fig. 1 and in the Methods section.

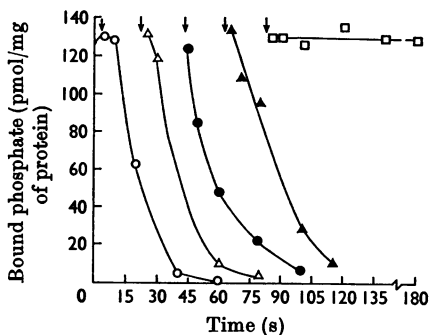


Fig. 9. Effect of detergents on the time-course at 37°C of the formation and decline of bound phosphate by the microsomal preparation. Additions to a final concentration of 0.1% (w/v) were made at 4s as indicated by the arrows. For clarity the test time-courses have been displaced 20, 40, 60 and 80s to the right. ○, Control (water); Δ , Lubrol W; ●, Triton X-100; \blacktriangle , sodium deoxycholate; \square , sodium dodecyl sulphate. All media contained 50 mM-NaCl and 2.5 μM -ATP. Other details are given in the legend to Fig. 1 and in the Methods section.

relatively high concentration of K⁺, but this was practically abolished when the sodium dodecyl sulphate concentration was increased to 7 mM (Fig. 10). Sodium decyl sulphate also stabilized the bound phosphate, but was five times less effective: a final concentration of 0.5% (20 mM) was required. Al-

though less effective the C₁₀ compound has the advantage of a much greater solubility below 4°C, enabling manipulation of labelled material to be carried out in the cold.

The relative effects of the sodium alkyl sulphates on the formation of bound phosphate and ATP hydrolysis were examined in greater detail. Both compounds inhibited ATP hydrolysis throughout the range of concentrations studied (Fig. 11). The concentration for half-maximal inhibition, K_1 , was 0.35 mM for sodium dodecyl sulphate and 2.0 mM for sodium decyl sulphate. A feature of the results is the stimulation of the formation of bound phosphate that was seen with both compounds in the range up to the concentrations giving half-maximal inhibition of hydrolysis (Fig. 11). At higher concentrations of detergent the formation of bound phosphate was rapidly inhibited, until at 3.5 mM and 20 mM, the respective concentrations of the C₁₂ and C₁₀ compounds found to stabilize the bound phosphate after formation, both phosphorylation and hydrolysis were completely inhibited. The effects of the detergents on enzyme activity were paralleled by their action in decreasing the turbidity of suspensions of the microsomes (Table 1). Here also the C₁₂ compound was about 6 times more effective than the C₁₀ compound.

The degree of stability conferred by the sodium alkyl sulphates was moderately good. At 37°C virtually no loss of label occurred over a period of 10 min. After gel filtration for 75 min on Sephadex

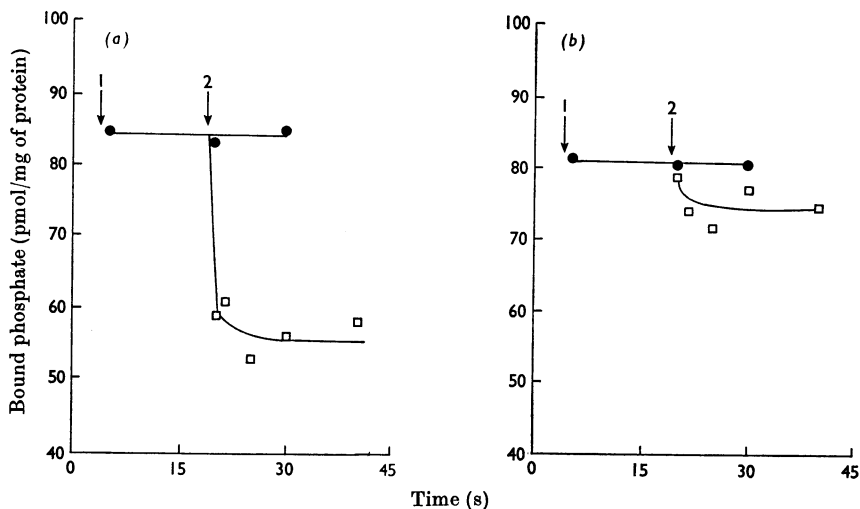


Fig. 10. Effect of K^+ on phosphate bound by the microsomal preparation after stabilization with sodium dodecylsulphate. KCl (\square) to 10 mM concentration was added at 19 s as indicated by arrow 2 after stabilization at arrow 1 with detergent. The final detergent concentration was: (a) 0.1%; (b) 0.2%. The medium contained 50 mM-NaCl and $2.5 \mu\text{M}$ -ATP. Other details are given in the legend to Fig. 1 and in the Methods section.

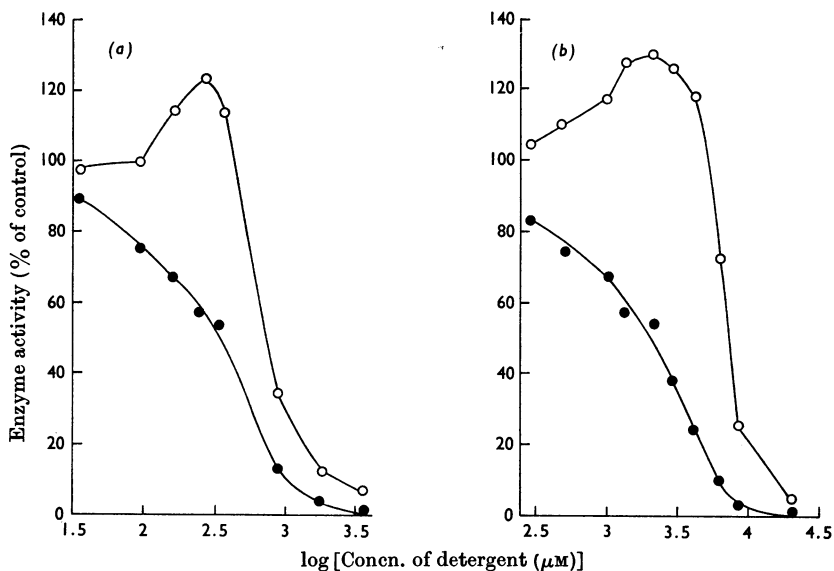


Fig. 11. Effect of (a) sodium dodecyl sulphate and (b) sodium decyl sulphate on the Na^+ -dependent formation of bound phosphate (O) and hydrolysis of ATP (●) in ox brain microsomes. The basal reaction mixture contained 50 mM-NaCl, $2.5 \mu\text{M}$ - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, $2.5 \mu\text{M}$ - MgCl_2 , 30 mM-tris-HCl buffer, pH 7.4 and $200 \mu\text{g}$ of membrane protein/ml. Incubation time was 5 s, the temperature 37°C .

G-25 at room temperature several preparations stabilized with sodium dodecyl sulphate retained in the protein peak from the column 25-47% of the

label found bound to the protein after the reaction had been stopped with acid. With 0.5% sodium decyl sulphate and by doing the gel filtration at 4°C

Table 1. *Effect of sodium alkyl sulphates on the turbidity of a microsomal suspension*

Microsomes (0.25 ml of a suspension containing 2 mg of protein/ml) were added to 2.25 ml of a medium containing 50 mM-NaCl, 30 mM-tris-HCl buffer, pH 7.4, 2.5 μ M-MgCl₂ and detergent as stated. After incubation for 10 min at 37°C the E_{540} of the suspension was determined (1 cm light-path).

Concn. of detergent (mM)	Turbidity (E_{540})	
	Sodium dodecyl sulphate	Sodium decyl sulphate
0	0.55	0.55
0.35	0.42	—
0.4	—	0.50
0.88	0.12	—
1.75	0.05	—
2.0	—	0.42
3.5	0.03	—
4.0	—	0.24
10.0	—	0.08
20.0	—	0.03

more than 50% of the label obtained by the usual technique was recovered (D. R. Alexander & R. Rodnight, unpublished work).

Thiol-blocking agents. Neither *p*-chloromercuribenzoate (to 0.2 mM) nor *N*-ethylmaleimide (to 1 mM) showed any appreciable effect on the rate of dephosphorylation when added to the full phosphorylated preparation. *p*-Chloromercuribenzoate initially accelerated the decline, but subsequently slowed it so that at 60 s the concentration of bound phosphate was about 15% higher than in the control. *N*-Ethylmaleimide slightly lowered the dephosphorylation rate without affecting the rate of ATP hydrolysis. Reaction of the latter compound with membrane preparations from nervous tissue is a time-dependent process (Skou & Hilberg, 1965; Fahn, Hurley, Koval & Albers, 1966b) and results in inhibition of dephosphorylation without affecting phosphorylation (Fahn *et al.* 1966b).

DISCUSSION

Time-course of formation of bound phosphate in the presence of Na⁺. The observation (Fig. 1) that the steady-state concentration of bound phosphate declines to less than 2% of its maximum as ATP hydrolysis approaches completion requires comment. Total dephosphorylation was not seen in the experiments of Rodnight *et al.* (1966) nor in those of Skou & Hilberg (1969), where as much as 20% of the bound phosphate appeared to be stable. Since Rodnight & Lavin (1966) have shown that most of the phosphate incorporated in the absence of added Na⁺ is relatively stable, it would appear that under conditions of very low ATP concentration the Na⁺-

dependent system in the new preparation utilizes virtually all the available ATP. No attempt therefore has been made to correct the values obtained for the Na⁺-dependent reaction for a Mg²⁺-dependent component, as this would clearly be invalid.

Action of chemical agents on the phosphorylated system. The various effects of the agents studied in this work and elsewhere can be grouped under four headings as follows.

(i) Agents that decrease the steady-state plateau concentration of Na⁺-dependent bound phosphate and simultaneously increase ATP hydrolysis. This is the typical action of certain univalent cations, of which K⁺ and NH₄⁺ are the prime examples, and is widely documented. [For observations on K⁺ see Rodnight *et al.* (1966), Nagano *et al.* (1967) and Skou & Hilberg (1969); a similar effect of NH₄⁺ on the kidney enzyme was described by Post, Sen & Rosenthal (1965); by using the present preparation and reaction conditions I found that 0.5 mM-ammonium chloride decreased bound phosphate by 30% and increased ATP hydrolysis by 40%.]

On the assumption that the bound phosphate is intermediary in ATPase action this type of response must stem from an increased turnover of the phosphorylated group through acceleration of reaction (3) given in the introduction. The reason why agents that increase turnover in this way decrease the plateau concentration of bound phosphate has never been adequately explained. The effect cannot be due to the phosphorylation reaction (2) being temporally more sensitive to denaturation than reaction (3), since the same effect of K⁺ is observed when the reaction is stopped by sodium dodecyl sulphate, a reagent that clearly affects primarily the dephosphorylation step. It is more probably a consequence of the limited quantity of ATP available reacting with fewer sites in the presence of K⁺ or NH₄⁺, a conclusion supported on the whole by the observation that as the ATP/protein ratio is raised the proportional effect of K⁺ is decreased (Skou & Hilberg, 1969; Rodnight, Carrera & Goldfarb, 1969).

(ii) Agents that decrease the plateau concentration of bound phosphate and partially inhibit ATP hydrolysis. In the present study suramin, calcium chloride and high concentrations of sodium chloride are examples in this category. Here the primary effect seems to be on phosphorylation, although inhibition of reaction (3) may also be occurring.

(iii) Agents such as ethanol and oligomycin that increase the plateau concentration of bound phosphate while partially inhibiting ATP hydrolysis. With ethanol nearly complete inhibition of hydrolysis is accompanied by an effect on phosphorylation as well.

(iv) The distinct action of the sodium alkyl sulphates. At the maximum concentrations used reactions (2) and (3) are both inhibited together

with ATP hydrolysis but since the plateau concentration of bound phosphate is maintained it must be assumed that inhibition of reaction (3) occurs marginally faster than that of reaction (2). The sodium alkyl sulphates therefore appear to exert their primary effect on the turnover of bound phosphate by inhibiting the dephosphorylation reaction. Assuming that ATP hydrolysis is proceeding through the turnover of bound phosphate it is evident that inhibition of hydrolysis occurs in two stages: at concentrations below the K_1 through inhibition of the K^+ -dependent dephosphorylation reaction and at concentrations above the K_1 through inhibition of phosphorylation as well. The action of these compounds is therefore similar to that of ethanol, which also inhibits dephosphorylation at low concentrations.

Further consideration must now be given to the action of certain of the agents used in this work.

Sodium chloride. The inhibition of phosphorylation by sodium chloride observed at concentrations of 150mM or higher was also noted in the cruder preparation (Rodnight *et al.* 1966), although in these experiments the Na^+ was present at the start of the reaction. In the electric-organ preparation obtained by Fahn *et al.* (1968), however, no inhibition by Na^+ was observed up to 500mM concentration with an ATP/protein ratio of 7.7nmol/ μ g as compared with 12.5pmol/ μ g used in the present study. The small inhibition of phosphorylation seen at 125mM-sodium chloride agrees with the hypothesis that turnover in the absence of added K^+ is due to traces of K^+ bound to the enzyme system, as proposed in the following paper (Goldfarb & Rodnight, 1970).

Ethanol. The remarkable resistance of the phosphorylation reaction to concentrations of ethanol at least five times those required to inhibit ATP hydrolysis (Israel *et al.* 1966) indicates that inhibition of the overall reaction is probably due to an interaction of the alcohol with the K^+ -binding site on the membrane; indeed, in the study by Israel *et al.* (1966) inhibition by ethanol was to some extent overcome by increasing the K^+ content of the medium. It is perhaps not surprising that ethanol and acetone appear to exert their major effect on reaction (3), since this involves the direct participation of water. It is conceivable, however, that the solvents are also acting by modifying the association of the enzyme system with a lipid.

Ouabain. The effect of ouabain on the phosphorylated enzyme does not fit into any of the four categories mentioned. The outstanding feature in the present experiments is the transient rise in the concentration of bound phosphate that immediately follows addition of the drug. This is surprising, since the inhibitory reaction of ouabain with the Na^+ , K^+ , Mg^{2+} -ATPase system is time-dependent, requiring in the presence of ATP at least 1min for full in-

hibition to develop (Albers, Koval & Siegel, 1968). Further, both in the present work and elsewhere (Skou & Hilberg, 1969; Albers *et al.* 1968) the drug has been found to inhibit phosphorylation at intervals of 5s or more after addition. This makes it unlikely that the transient rise is due to an acceleration of phosphorylation. It must be concluded therefore that ouabain initially reacts very rapidly with the dephosphorylation site to cause enough inhibition of reaction (3) to permit the concentration of bound phosphate to rise, but that a few seconds later it starts to inhibit the phosphorylation site, causing the concentration of bound phosphate to fall to a lower plateau concentration, the value of which is related to the degree of inhibition of hydrolysis. The incomplete inhibition of ATP hydrolysis under these conditions may be partly due to a transfer of ATP to the Mg^{2+} -ATPase system and partly to incomplete binding of ouabain over the short time-interval (1min) observed. The fact that the low concentration of bound phosphate that persists after addition of the drug is slowly turning over, as shown by its eventual decline to zero, indicates that some of the residual activity is due to the Na^+ , K^+ , Mg^{2+} -ATPase system. It may be noted that the complex effect of ouabain observed here may bear little relation to its effect on the cation-transport mechanism in the intact cell, since in suspensions of cell membrane fragments the drug presumably has simultaneous access to both sites on the enzyme system. In the intact cell, on the other hand, it is reasonable to assume that the drug reacts first and foremost with the K^+ -binding site on the outside of the membrane, and the extent to which the very low concentrations required to inhibit cation transport penetrate the cell is questionable.

Inasmuch as the effects of the different agents studied in this work can be reasonably interpreted in terms of the reaction sequence given in the introduction to this paper, the results reinforce the view that the Na^+ -dependent phosphorylation reaction functions as a direct intermediate in the hydrolysis of ATP by the Na^+ , K^+ , Mg^{2+} -ATPase system. The results also serve to emphasise the complexity of the ATPase system, especially the spatial separation of the Na^+ - and K^+ -binding sites as shown by the different sensitivities of the phosphorylation and dephosphorylation reactions to the agents studied.

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