Phosphate Binding by Cerebral Microsomes in Relation to Adenosine-Triphosphatase Activity

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1. Microsomes prepared from guinea-pig and ox brain were incubated for periods of a few seconds with low concentrations of $\text{Mg}\cdot[^{32}\text{P}]\text{ATP}$, the reaction was stopped with trichloroacetic acid and determinations were made of the phosphate bound to the acid-washed, and in some cases solvent-extracted, residue. 2. At $20\text{ }\mu\text{M}\cdot\text{ATP}$, at 37° and in the presence of Na^+ ions, 30–50 μmoles of phosphate/mg. of microsomal protein were bound by the preparation within 1 sec. of starting the reaction; little further change in level occurred until hydrolysis of ATP exceeded 50%, when the bound phosphate began to decline fairly rapidly to the zero-time value. 3. At $20\text{ }\mu\text{M}\cdot\text{ATP}$ without Na^+ ions present or in the presence of K^+ ions, the level of bound phosphate increased gradually and did not decline as ATP hydrolysis approached completion. 4. Potassium ions either inhibited the formation of Na^+ -dependent bound phosphate or, when added during the course of the reaction, rapidly reduced its level. 5. At $200\text{ }\mu\text{M}\cdot\text{ATP}$ the bound phosphate formed in the presence of Na^+ ions appeared to consist of a mixture of the unstable Na^+ -dependent type and the stable type requiring only Mg^{2+} ions for its formation. 6. Non-radioactive ATP added during the course of the reaction at $20\text{ }\mu\text{M}\cdot\text{ATP}$ with Na^+ ions present rapidly discharged virtually all the bound ^{32}P counts; at $200\text{ }\mu\text{M}\cdot\text{ATP}$ only a proportion of the label was similarly discharged. The Na^+ -dependent bound phosphate is therefore turning over, in contrast with that formed in the absence of Na^+ ions, which proved more stable. 7. The Na^+ -dependent bound phosphate was not in the form of ATP; experiments with $[^{14}\text{C}]\text{ATP}$ instead of $[^{32}\text{P}]\text{ATP}$ showed a small and invariable binding of ATP by the preparation unaffected by Na^+ ions or time of incubation. 8. Under the usual conditions employed in this work ouabain stimulated formation of Na^+ -dependent bound phosphate when Na^+ ions were suboptimum and inhibited it when optimum Na^+ ions were present. 9. The Na^+ -dependent binding reaction under present conditions did not involve incorporation into phosphorylserine groups. 10. The relation of the findings to the $(\text{Na}^+, \text{K}^+)\cdot\text{ATPase}$ of the preparation, and to observations in brain slices appearing to implicate phosphorylserine groups in cation transport, is discussed.

In investigating by isotopic methods the functional significance of protein-bound phosphorylserine located in cerebral membranes, and in particular their possible relevance to the $(\text{Na}^+, \text{K}^+)\cdot\text{ATPase}$ * and to cation transport, Rodnight & Lavin (1966) observed incorporation of the γ -phosphate of ATP into intrinsic phosphoproteins of guinea-pig brain microsomes. A feature of these experiments was the employment of $[^{32}\text{P}]\text{ATP}$ concentrations in the intracellular range (1–3 mM) and incubation times of a few minutes. These are usual conditions for determining ATPase activity in membrane preparations (Schwartz,

Bachelard & McIlwain, 1962) and might have yielded evidence for the participation of a phosphoprotein in the reaction if the protein constituted the only major phosphate acceptor in the preparation. Rather than ATP reacting with one acceptor molecule, however, it appeared that binding of phosphate, and also incorporation into phosphorylserine groups, was occurring simultaneously in different molecular structures in the membrane; moreover, the mean rate of phosphate transfer was only 0.1% of the rate of ATP hydrolysis and the process appeared to be inhibited by Na^+ , K^+ and Li^+ ions. It was concluded therefore that the binding observed under these conditions was not directly related to ATPase activity.

An alternative approach used by several other

* Abbreviations: ATPase, adenosine triphosphatase; $(\text{Na}^+, \text{K}^+)\cdot\text{ATPase}$, Na^+ -plus- K^+ -stimulated adenosine triphosphatase.

groups of workers (Albers, Fahn & Koval, 1963; Post, Sen & Rosenthal, 1965; Gibbs, Roddy & Titus, 1965; Skou, 1965; Hokin, Sastry, Galsworthy & Yoda, 1965; Nagano *et al.* 1965) and by ourselves in a preliminary study (Rodnight & Lavin, 1964), has been to observe the transfer of phosphate to membrane components in the presence of very low concentrations of ATP and over incubation times of a few seconds, in the expectation that an intermediate in the ATPase system would exhibit high turnover and affinity for ATP and therefore become preferentially labelled in the initial stages of the reaction. Experiments which appear to confirm this expectation were first reported by Charnock & Post (1963): Na⁺ ions were observed to stimulate the labelling of a kidney membrane preparation by [³²P]ATP (20 μM), whereas low concentrations of K⁺ ions added to the same system had the opposite effect. The data were interpreted as consistent with a particular two-stage mechanism for the membrane ATPase enzyme, as earlier proposed by Skou (1960) and Ahmed & Judah (1962). In the literature cited above similar findings have since been described for other membrane systems. The present paper describes an application of this experimental approach to the microsomal preparation from guinea-pig cerebral cortex that contains a phosphoprotein sensitive to the effects of electrical pulses applied to the intact tissue (Trevor & Rodnight, 1965); observations employing a similar preparation from ox brain are also included.

EXPERIMENTAL

Materials

All chemicals were of reagent grade. Solutions were made with deionized water of resistance greater than 4 megohms/cm. at 16°. ATP and ADP were obtained as the sodium salts from Sigma Chemical Co., St Louis, Mo., U.S.A. Where required the tris salt of ATP was prepared by chromatography on Dowex 50 (X12) (see Rodnight & Lavin, 1966). [8-¹⁴C]ATP (sodium salt, specific radioactivity 4.8 mc/mmole) and carrier-free [³²P]P_i (for [³²P]ATP preparation) were obtained from The Radiochemical Centre, Amersham, Bucks.

Preparations

Microsomes. Membrane-rich fractions were prepared from both guinea-pig and ox brain by the procedure of Rodnight & Lavin (1966). Dispersions of the guinea-pig material in the sucrose medium were usually made within 30 min. of killing the animal. Ox brain from a slaughterhouse was brought to the laboratory sealed in plastic bags surrounded by crushed ice. Dissection commenced about 2 hr. after death and the dispersion was made within 4 hr. Grey matter was obtained by cutting away small portions of cerebral cortex from the underlying white matter with scissors, taking suitable precautions to maintain the tissue

at 4° or less throughout. The separated fragments of grey matter were immersed in a known weight of sucrose medium (0.32M-sucrose containing 0.5 mM-EDTA brought to pH 7 with tris) pending dispersion in a glass-Teflon homogenizer. The yield of microsomal protein from the ox-brain source ranged from 12 to 18 mg./g. of cerebral cortex. The preparations were stored in 0.32M-sucrose (containing 0.5 mM-EDTA at pH 7) at -20° at a concentration of 5 mg. of protein/ml. The procedure used for determining (Na⁺, K⁺)-ATPase activity in the preparations and rates obtained with the guinea-pig material are given in the preceding paper (Rodnight & Lavin, 1966). In the ox-brain preparations specific activities were in the range 28-54 and 11-23 μmoles of P/mg. of protein/hr. in the presence and absence of the univalent cations respectively. In all preparations the enzyme activity was stimulated by Na⁺ approx. threefold. [³²P]ATP. The enzymic procedure for the γ-labelled product already described was used (Rodnight & Lavin, 1966).

Procedures

Protein. This was determined by a modification of the biuret method of Gornall, Bardawill & David (1949), with bovine plasma albumin as standard.

Reaction conditions. The initial series of experiments were carried out in thin-walled plastic centrifuge tubes (reaction volume 30 ml.). The usual reaction mixture contained tris-HCl buffer (pH 7.5, 30 mM), the tris salt of [³²P]ATP (20 μM, 1-4 × 10⁷ counts/min./μmole of γ-P), MgCl₂ (20 μM) and microsomal protein (2 mg.). The mixture, without [³²P]ATP and microsomes, was first equilibrated at 37°; adequate mixing was provided by an electrically driven glass stirrer. After addition of [³²P]ATP the reaction was started by rapid injection of the microsomes and was allowed to proceed for the desired time before stopping with 2 ml. of 100% (w/v) trichloroacetic acid. Immediately after addition of the latter, 0.1 ml. of 2% (w/v) bovine plasma albumin was added to assist the precipitation of the dilute microsomal protein; the added carrier protein was dissolved by the first ethanol wash and its use did not affect the results. The contents of the tubes were rapidly cooled by immersion in solid CO₂-methylated spirits and then centrifuged for 25 000g-min. After decantation [³²P]P_i in the supernatants and bound ³²P in the precipitates were determined by the procedures given below.

For the majority of experiments it proved more convenient to adopt a reaction volume of 2.5 ml. The composition of the reaction mixture was identical to that used in other experiments, except that only 1 mg. of microsomal protein was added. The reaction was carried out in polypropylene centrifuge tubes (100 mm. × 13 mm.), and the contents were magnetically stirred by plastic-covered bars. To start the reaction the microsomes, in a volume of 0.2 ml., were injected below the surface of the vigorously stirred medium with a 1 ml. syringe fitted with 10 cm. of narrow-gauge polythene tubing. The reaction was stopped by the addition also from a syringe, of 1.25 ml. of ice-cold 10 or 15% (w/v) trichloroacetic acid; a further 1.25 ml. of acid was then used to wash the stirrer bar. The tubes were cooled in ice before centrifuging and proceeding as indicated below.

In both systems observations with coloured solutions showed that stirring was adequate to ensure complete admixture of the microsomes within approx. 1 sec. of addition. Data quoted for experiments of 1 sec. duration are, of

course, subject to a considerable error and are reported only to illustrate the very rapid nature of the binding reaction.

Bound P determination. In experiments on the 30 ml. scale the protein precipitates were dispersed in approx. 3 ml. of 10% trichloroacetic acid and transferred quantitatively to small polypropylene centrifuge tubes. They were then washed with 10% trichloroacetic acid, ethanol and chloroform-methanol (2:1, v/v) as described by Rodnight & Lavin (1966). Precipitates from experiments on the 2.5 ml. scale were similarly treated, except that the 10% trichloroacetic acid contained $m\text{-H}_3\text{PO}_4$, and in later experiments four rather than three washes with acid were employed. The washed precipitates were dissolved in $n\text{-NaOH}$ and counted in a M6H liquid counter (20th Century Electronics, New Addington, Surrey). The effect of repeated washing of the precipitate with the trichloroacetic acid- H_3PO_4 reagent is shown in Table 1: the difference between using three and four washes was small but nevertheless sufficient to justify the longer procedure. During the acid-washing procedure it was found very important to maintain the precipitate at all times at 0° and to complete the procedure as rapidly as possible. The necessity for these rigorous conditions was made clear in a later study of the lability of the phosphate bound under these conditions (Hems & Rodnight, 1966), where the major part was found to be readily released as P_i in aqueous suspensions by a time-, pH- and temperature-dependent process. At low pH and 0° , however, the bound phosphate was adequately stable, as can be seen from Table 1.

In most experiments on the 2.5 ml. scale the washes with ethanol and chloroform-methanol were omitted since it was found that at low ATP concentrations the solvents removed only 10–15% of the total bound P.

ATP hydrolysis by P_i determination. The following isotopic procedure was employed. The first acid supernatants were decanted into glass-stoppered tubes graduated at 10 ml. and containing sufficient $n\text{-NaOH}$ to bring the pH to 6–8. The volume was made to 10 ml. with water and 1 ml.

transferred to another stoppered tube containing ATP (0.5 μmole) and KH_2PO_4 (0.25 μmole) in 1 ml. After addition of 2-methylpropan-1-ol-benzene (1:1, v/v; 2.5 ml.), $10N\text{-HClO}_4$ (0.1 ml.) and 5% (w/v) ammonium molybdate (0.5 ml.), the tube was immediately shaken for 20 sec. and then centrifuged briefly. A portion of the organic layer, usually 0.1 ml., was diluted to 10 ml. with acid ethanol and counted in a M6H liquid counter.

ATP hydrolysis by ADP determination. The reaction was carried out on the 2.5 ml. scale with [^{14}C]ATP instead of [^{32}P]ATP. The first acid supernatant was extracted six times with equal volumes of diethyl ether to remove trichloroacetic acid, and immediately fractionated on a column (0.9 cm. $\times$ 3.0 cm.) of Dowex 1 (X8; 200–400 mesh; formate form). After washing the column with 5 ml. of water AMP, ADP and ATP were respectively eluted by the following three reagents: (1) 0.2N-formic acid (60 ml.), (2) 4N-formic acid, 0.05M in ammonium formate (45 ml.) and (3) 4N-formic acid, 0.5M in ammonium formate (40 ml.). The eluates were concentrated by freeze-drying and portions plated on stainless-steel planchets and counted in a thin window gas-flow automatic scaler (Nuclear-Chicago Corp., Chicago, Ill., U.S.A.).

Determination of bound adenine. The precipitates from the [^{14}C]ATP experiments, including ^{32}P -labelled precipitates for comparison, were washed three times with the trichloroacetic acid- H_3PO_4 reagent in the usual manner and then, successively, still at 0° , with ethanol-ether (1:1, v/v), chloroform-methanol (2:1, v/v) and ethanol-ether (1:1, v/v). They were then dissolved in a small volume of 98% (w/w) formic acid and transferred quantitatively to stainless-steel planchets. After drying with radiant heat the samples were counted in the gas-flow scaler.

General comments on procedure. In each experiment the specific radioactivity of the appropriate sample of [^{32}P]ATP was determined separately and used to calculate the amounts of γ -phosphate bound or released as P_i . Zero-time controls were carried out by adding the usual amount of trichloroacetic acid to the reaction mixture before injection of the microsomes. Experimental points for both the binding reaction and the ATPase were normally established in duplicate and with a few exceptions the results quoted are mean values. Duplicate values for the Na^+ -dependent bound P rarely varied by more than $\pm 10\%$ of the mean; the variation tended to be greater for binding observed in the absence of Na^+ (which did not exhibit the plateau observed in the former case) and for the ATPase reactions.

RESULTS

Time-course of the binding reaction

With 20 μM -ATP. At 37° and with Na^+ present the initial rate of phosphate binding was very rapid, reaching a maximum 25–45 μmoles of P/mg. of protein within about 1 sec. of starting the reaction and then remaining steady at this level for at least 10 sec. In the absence of Na^+ , or with K^+ present, the level of bound P increased gradually over an initial period, ranging in different experiments from 10 to 16 sec., to a final level which was always lower than the maximum attained with optimum Na^+ in the reaction mixture. These features of the first

Table 1. *Stability of bound phosphate to washing with trichloroacetic acid*

Several samples of guinea-pig microsomes were incubated with [^{32}P]ATP and 15 mM-NaCl for 10 sec. at 37° as described in the text (2.5 ml. scale). After precipitation with trichloroacetic acid the denatured microsomes were separated by centrifuging and washed at 0° from three to eight times in the centrifuge with 10% (w/v) trichloroacetic acid containing $m\text{-H}_3\text{PO}_4$. Counts in the supernatants were determined from the first wash and in the precipitates after three washes.

Number of washes	Phosphate (μmoles)	
	Supernatant	Precipitate
1	740	—
2	18	—
3	4	23
4	3	19
5	<2	18
6	<2	17
7	<2	16
8	<2	16

Fig. 1. Time-course of bound P formation and ATP hydrolysis at 37° by guinea-pig microsomes with 20 μ M-ATP. Details of the reaction systems and determinations of P_i and bound P are given in the text. The first 10 sec. of the reaction are shown on the left (a) and a longer time-course for experiments on the 2.5 ml. scale on the right (b). ○, ●, △ and ▼ refer to observations made in the 2.5 ml. system on the same microsomal preparation (the precipitates were not treated with solvents): time-course △, with Mg²⁺ as only added cation; ●, with 15 mM-NaCl; ○, with 100 mM-NaCl; ▼, with 15 mM-KCl. The remaining symbols refer to an experiment carried out in the 30 ml. system with a different preparation and where the precipitates were treated with solvents: time-course ▲, with Mg²⁺ only; □, with 15 mM-NaCl; ■, with 150 mM-NaCl. Broken lines indicate rate of ATP hydrolysis, the abscissa for which has been displaced 1 min. to the right for clarity.

10 sec. of the reaction at 37° with 20 μ M-ATP are illustrated in Fig. 1(a); they have been consistently observed in many experiments on the 2.5 ml. and 30 ml. scales, with guinea-pig brain and ox-brain microsomes, and in several respects they resemble results obtained by other workers using different membrane preparations (Post *et al.* 1965; Gibbs *et al.* 1965; Ahmed & Judah, 1965; Skou, 1965). The subsequent course of the reaction on the 2.5 ml. scale is given in Fig. 1(b), which shows that the level of the Na⁺-dependent binding began to decline after about 50% of the ATP had been hydrolysed and eventually approached control levels. On the 2.5 ml. scale this occurred after approx. 10 sec. of incubation at 37° and the downward slope of the resulting curve crossed the time-course of the slower binding reaction between 20 and 50 sec. from zero time.

Because of the very rapid time-course of the binding a comparison of the initial rates of bound P formation and ATP hydrolysis at 37° was not possible. An approximate comparison could, however, be made at 0°, where the sharp inflexion in the time-course of the Na⁺-dependent binding was

Fig. 2. Time-course of formation of bound P and ATP hydrolysis by guinea-pig and ox-brain microsomes at 0°, with 20 μ M-ATP on the 2.5 ml. scale. Solvent treatment was not applied; other procedural details were as described in the text. □, Time-course at 0° for guinea-pig preparation with 15 mM-NaCl present. The remaining points refer to an ox-brain preparation suspended in EDTA-free sucrose. Time-course △, with Mg²⁺ as only added cation; ●, with 15 mM-NaCl; ○, with 100 mM-NaCl.

absent (Fig. 2). With optimum sodium chloride (100 mM) present the minimum initial rate of total bound P formation under these conditions was 300 μ moles of P/min. compared with a figure of at least 3000 μ moles of P_i/min. for the total ATPase activity under the same conditions. It is also worth noting that the Na⁺-dependent bound P appeared more stable at this temperature.

With 200 μ M- and 1 mM-ATP. When the ATP concentration was raised tenfold in the absence of Na⁺ the phosphate binding increased markedly to a maximum level (reached after some 3 min. of incubation) which was nearly 20 times higher than that observed with 20 μ M-ATP. Addition of Na⁺ to the 200 μ M system stimulated the binding rate over the first minute of incubation, but subsequently the Na⁺-dependent course levelled off to a maximum which was only 10 times that found with the same concentration of Na⁺ and 20 μ M-ATP (Fig. 3). Thus the eventual effect of Na⁺ was one of inhibition, as reported in the previous paper for observations at 3 mM-ATP concentration (Rodnight & Lavin, 1966). Further, the bound P formed with 200 μ M-ATP in the presence of Na⁺ was more stable than that produced with Na⁺ present at the lower substrate concentration: the rapid fall in bound P observed at

Fig. 3. Time-course of bound P formation and ATP hydrolysis at 37° by guinea-pig microsomes with 200 μ M- and 1 mM-ATP. With the exception of the higher ATP concentrations and $MgCl_2$ concentrations (maintained equimolar with ATP) the procedures were as given in the text for the 2.5 ml. system; the precipitates were not treated with solvents. The time-course for the first minute is shown on the left and over a period of 20 min. with 200 μ M-ATP on the right. Time-course $\square$, with 1 mM-ATP and Mg^{2+} as only cation; $\blacksquare$, with 1 mM-ATP and 15 mM-NaCl; $\circ$, with 200 μ M-ATP and Mg^{2+} only; $\bullet$, with 200 μ M-ATP and 15 mM-NaCl.

20 μ M-ATP after hydrolysis of 50% of the ATP was absent and even when the ATP concentration was zero only a relatively slow decline ensued. Evidently at the higher ATP concentration a greater proportion of the binding was of the more stable type not requiring Na^+ . At 1 mM-ATP concentration there was further enhancement of the binding reaction not requiring Na^+ and the time-courses with and without Na^+ crossed after only 10 sec. incubation at 37° (Fig. 3).

Effects of cations

Mg^{2+} and Ca^{2+} . With microsomes suspended in an EDTA-free sucrose medium neither the binding nor the ATPase showed an absolute requirement for Mg^{2+} on the 2.5 ml. scale with 20 μ M-ATP: moreover, in the absence of any added Mg^{2+} , Na^+ slightly stimulated the two reactions (Table 2). The residual activity not requiring added Mg^{2+} was virtually abolished by EDTA, showing that it was almost certainly due to Mg^{2+} associated with the microsomal suspension. In the 2.5 ml. system as normally employed the EDTA incorporated in the sucrose medium was adequate to chelate this intrinsic Mg^{2+} , and also part of the added Mg^{2+} . For example, in an experiment in which microsomes from the same source, suspended either in 0.32M-sucrose or in the usual sucrose medium containing EDTA, were compared, the ATP split

Table 2. *Effect of Mg^{2+} on bound phosphate formation and ATP hydrolysis*

Guinea-pig microsomes, suspended in 0.32M-sucrose, were incubated on the 2.5 ml. scale with [^{32}P]ATP (20 μ M) for 5 sec. at 37°; solvent treatment was not applied. EDTA was added as the tris salt at pH 7. Other procedural details were as described in the text.

Additions			Bound P (μ moles/mg. of protein)	ATP split (%)
Mg (μ M)	Na (mM)	EDTA (mM)		
0	0	0	9	16
10	0	0	14	17
20	0	0	19	22
0	15	0	16	20
10	15	0	21	30
20	15	0	23	36
0	15	0.04	6	3
0	15	0.10	5	2
20	15	0.04	13	12
20	15	0.10	8	3
20	15	10	1	0

Table 3. *Effect of EDTA on rate of decline of bound phosphate*

Guinea-pig microsomes were incubated with [^{32}P]ATP (20 μ M) in the presence of 15 mM-NaCl at 37° in the 2.5 ml. system. Solvent treatment was not applied; other procedural details were as described in the text. Where appropriate 25 μ l. of m-EDTA (as tris salt at pH 7) was added 10 sec. after starting the reaction. Results from two experiments are given.

Time (sec.)	Bound P (μ moles/mg. of protein)	ATP split (%)
10	27, 31	47, 56
20	22, 28	75, 73
20, EDTA at 10	12, 16	48, 55

within 10 sec. with 15 mM-NaCl present was 46% in the latter medium compared with 64% in the plain sucrose medium; the corresponding increase in total bound P was 24%. However, although the sub-optimum levels of Mg^{2+} on the 2.5 ml. scale as normally carried out resulted in lower values for bound P, they did not affect the Na^+ -increment in the binding; if anything, they tended to enhance this by depressing bound P formation in the absence of added Na^+ . On the 30 ml. scale both the EDTA and microsomal-derived Mg^{2+} presumably were sufficiently diluted to render their effect negligible, accounting for the higher ATPase specific activity found under these conditions (Fig. 4).

To determine the Mg^{2+} -dependence of the reaction releasing bound P excess of EDTA was added to the reaction mixture after labelling for

Table 4. *Effect of an increased concentration of Mg²⁺ on bound phosphate formation and ATP hydrolysis*

Guinea-pig microsomes were incubated with [³²P]ATP (20 μM) at 18° on the 2.5 ml. scale, with Na⁺ and Mg²⁺ added as indicated. The acid-washed precipitates were further treated with ethanol and chloroform-methanol and total counts determined in the unwashed solvent extract. Other procedural details were as described in the text.

Cations (mM)	Bound P (μmoles/mg. of protein)		ATP hydrolysis (mμmoles of P/mg. of protein)		P extracted by solvents (μμmoles/mg. of protein)	
	1sec.	10sec.	1sec.	10sec.	1sec.	10sec.
	1sec.	10sec.	1sec.	10sec.	1sec.	10sec.
Mg ²⁺ , 0.02	7	9	2	7	2	2
Mg ²⁺ , 0.02; Na ⁺ , 15	21	21	3	12	2	3
Mg ²⁺ , 0.2	13	27	3	17	3	14
Mg ²⁺ , 0.2; Na ⁺ , 15	24	33	4	23	4	13

Fig. 4. Effect of Na⁺ concentration on bound P formation and ATP hydrolysis at 37° by guinea-pig microsomes with 20 μM-ATP. Reaction time was 10 sec. Other procedural details were as given in the text. Open symbols refer to results obtained on the 2.5 ml. system where the precipitates were not treated with solvents; filled-in symbols refer to results on the 30 ml. scale where the solvent treatment was applied. All results are corrected for zero-time controls.

Increasing the ratio ATP:Mg²⁺ in the reaction mixture from 1:1 to 1:10 depressed the Na⁺ increment in the binding, but had no effect on the corresponding increment in the ATPase reaction. In the absence of Na⁺, on the other hand, a tenfold increase in Mg²⁺ concentration stimulated and prolonged the binding, and also increased the counts recovered in a solvent extract of the precipitated microsomes, presumably due to labelled phospholipids. These results are illustrated for an experiment at 18° in Table 4.

Both the binding and the ATPase reaction were inhibited by Ca²⁺. The effect on the Na⁺-dependent binding was discernible in the 2.5 ml. system at 5 μM-calcium chloride, and at 50 μM-calcium chloride the bound P formed in 5 sec. was reduced by 50%.

Na⁺ and K⁺. The formation of bound P with 20 μM-ATP was more sensitive to Na⁺ when measured in the 30 ml. system than in the 2.5 ml. system; in the former maximum binding at 10 sec. required only 15 mM-sodium chloride as against 100 mM-sodium chloride in the latter (Fig. 4). ATP hydrolysis, on the other hand, was maximally activated in both systems in the range 5–15 mM-sodium chloride, with higher concentrations of Na⁺ progressively inhibiting the reaction. Inhibition of bound P formation by high Na⁺ required concentrations of 200 μM or above; even at 0.5 M-sodium chloride the bound P level was twice that found without Na⁺, although the ATPase activity at this concentration of Na⁺ was virtually zero.

Except for a higher maximum level and a slower decline over the period 10–30 sec., bound P formation in the 2.5 ml. system followed a similar time-course in media containing 15 and 100 mM-sodium chloride (Fig. 1). The slower decline was presumably related to the reduced ATPase activity at 100 mM-sodium chloride.

The lower sensitivity of the binding reaction to Na⁺ on the 2.5 ml. scale was also observed with

10 sec. in the presence of Na⁺. Stabilization of the label as a result of this addition would have suggested bivalent cation dependence for the dephosphorylation step. However, EDTA greatly enhanced the rate of loss of bound P over the period 10–20 sec. (Table 3), showing that the releasing reaction is independent of Mg²⁺ reacting with EDTA. An essentially similar finding with a different preparation of ox-brain microsomes has been reported by Gibbs *et al.* (1965).

200 μM -ATP and ox-brain microsomes. It did not appear to be related to Mg^{2+} concentrations (which, as explained above were suboptimum in the usual 2.5 ml. system at 20 μM -ATP concentration) but rather to a difference in enzyme protein concentration in the two systems. The two concentrations were 0.07 and 0.4 mg. of protein/ml. of reaction mixture for the 30 and 2.5 ml. systems respectively; when experiments on the smaller scale were conducted with diminishing amounts of microsomal protein, the sensitivity of the binding reaction to Na^+ progressively increased, so that at 0.1 mg./ml. maximum sensitivity was approached at 15 mM concentration. This point has not been investigated further but from the data available it seems that the sensitivity of the binding reaction to Na^+ is determined by the ratio of Na^+ to microsomal material in the reaction mixture, as well as by the Na^+ concentration.

The Na^+ -dependent binding at 20 μM -ATP was very sensitive to K^+ . Fig. 5(a) shows that inclusion of 0.5 mM-potassium chloride in the reaction mixture approximately halved the amount of bound P formed in 5 sec. in the presence of either 15 or 100 mM-sodium chloride. Higher concentrations of K^+ , however, did not entirely inhibit bound P formation, although the level reached in 5 sec. with 5 mM-potassium chloride was rather less than expected if Na^+ had been absent from the reaction

mixture. Sensitivity of the preparation to K^+ could also be satisfactorily demonstrated after establishment of the steady-state level of Na^+ -dependent bound P, both at 37° and 16° (Fig. 5b).

Sensitivity of the ATPase system to Na^+ and K^+

This requires special consideration because of the unusual conditions under which the splitting of ATP was observed in this work, namely high enzyme and low substrate concentrations. These conditions were necessary for the determination of bound P formation with the facilities available and it was recognized they would probably modify the usual characteristics of the ATPase reaction. With regard to Na^+ , the sensitivity of the present system followed the pattern now well established in more conventional assay systems. Thus in a K^+ -free medium over the range 0–100 mM-sodium chloride, especially on the 30 ml. scale, the sensitivity was similar to that described by Skou (1962) for a different brain preparation (Fig. 4). As has been already pointed out, Na^+ -sensitivity on the 2.5 ml. scale was rather low, but this appeared to be mainly due to a suboptimum Mg^{2+} concentration. A further point regarding the 2.5 ml. system requiring comment is the finding that concentrations of Na^+ higher than 100 mM continued to inhibit the activity to levels well below those found without added Na^+ .

Fig. 5. Effect of K^+ on bound P formation and ATP hydrolysis by guinea-pig microsomes with 20 μM -ATP. (a) Effect of K^+ concentrations. Reaction time was 5 sec., temp. 37°. ○, Results with 100 mM-NaCl; ●, the same preparation with 15 mM-NaCl; □, another preparation with 15 mM-NaCl. (b) Effect of 0.5 mM-KCl added during the course of the reaction. Broken lines indicate the time-course of bound P formation after addition (shown by arrows) of 1.25 μmole of KCl 3 sec. after starting the reaction in the presence of 15 mM-NaCl. ○, Reaction temp. 37°; □, temp. 16°. Other details were as given in the text.

This suggested that the system was partially activated, in the absence of added univalent cations, by traces of intrinsic Na⁺ and K⁺, and that the high concentrations of added Na⁺ were displacing the intrinsic K⁺ from its sites. So far as Na⁺ is concerned, we could find no definite evidence for this assumption: determinations of Na⁺ in the reagents and in several different preparations showed that the maximum contribution from these sources amounted only to 0.1 μ mole of Na⁺/ml. of reaction mixture, of which some 90% was derived from the microsomes. It remains possible, however, as Järnefelt (1964) pointed out, that this small quantity of intrinsic Na⁺ partially activates the ATPase system because it is bound in a specific manner to the enzyme sites.

Sensitivity of the ATPase reaction to K⁺ under the present conditions of high enzyme and low ATP concentrations was rather low, as can be seen from Fig. 5(a). With 15mM-sodium chloride present, addition of K⁺ gave no further activation, but at 100mM-sodium chloride concentration addition of 0.5mM-potassium chloride restored the activity to levels close to those observed with 15mM-sodium chloride alone. This again suggested the presence of enough intrinsic K⁺ in the system to stimulate the reaction at 15mM-sodium chloride, but insufficient to displace Na⁺ from K⁺ sites at 100mM-sodium chloride concentration. Determination of K⁺ in the reagents and microsomes suggested that this interpretation was feasible, since the mean value found in three instances was 0.12 μ mole of K⁺/ml. of reaction mixture, which is within the range over which added K⁺ acts on the Na⁺-dependent binding system. About 40% of the intrinsic K⁺ could be removed by centrifugal washing of the microsomes, but the washed preparation exhibited the same sensitivity to K⁺ as before, suggesting that the activating K⁺ was also specifically bound to enzyme sites.

Binding of [¹⁴C]adenine from [¹⁴C]ATP by microsomes

The results described above gave no indication of the possible nature of the bound phosphate. One possibility that merits consideration in addition to that of phosphoryl group transfer is the binding of ATP itself. To investigate this point the binding of the adenine moiety of [8-¹⁴C]ATP by ox-brain microsomes was examined. To confirm the enzymic activity of the enzyme preparation control experiments with [³²P]ATP were carried out in parallel under identical conditions. Further, since the [¹⁴C]ATP was obtained from a different source to the [³²P]ATP it was necessary to confirm that it constituted a satisfactory substrate for the ATPase; this was done by determining chromatographically

[¹⁴C]ADP and [¹⁴C]AMP in the first acid supernatants.

All observations were made in the 2.5ml. system with 20 μ M-ATP. Binding of adenine carbon atoms amounted to 3–4 μ moles/mg. of protein (expressed in terms of original nucleotide base) under all experimental circumstances, that is in the zero-time controls or after incubation in the presence or absence of Na⁺, regardless of the duration of the reaction. By contrast, the binding of P from the [³²P]ATP exhibited the usual time-course and Na⁺-dependence (Table 5). Hydrolysis of the [¹⁴C]ATP was satisfactory: after 20sec. of incubation approx. 7% of the total nucleotide was recovered as AMP and 90% as ADP, confirming a similar rate to that of the [³²P]ATP and also showing that the preparation contained a satisfactorily low level of adenylate

Table 5. *Binding of ¹⁴C from [¹⁴C]ATP by ox-brain microsomes*

Ox-brain microsomes were incubated at 37° with [¹⁴C]-ATP or [³²P]ATP (20 μ M) in the presence or absence of 15mM-NaCl. The conditions of the 2.5ml. system were employed. After the reaction had been stopped with trichloroacetic acid, precipitates were counted in a gas-flow counter as described in the text. Results are derived from two experiments with the same preparation.

Cations (mM)	Reaction time (sec.)	Labelling (μ moles/mg. of protein)	
		³² P	¹⁴ C
Mg ²⁺ , 0.02	0	4	3
	10	12	4
	60	15	4
Mg ²⁺ , 0.02; Na ⁺ , 15	0	5	4
	3	28	4
	10	32	3
	60	11	3
	180	9	3

Table 6. *Time-course of hydrolysis of [¹⁴C]ATP by ox-brain microsomes*

Ox-brain microsomes were incubated at 37° with [¹⁴C]-ATP (20 μ M) in the presence of 15mM-NaCl in the 2.5ml. system. After the reaction had been stopped with trichloroacetic acid, nucleotides were fractionated on Dowex 1 (X8) and counted as described in the text. Values quoted are the mean of two experiments conducted with the same preparation.

Time (sec.)	Nucleotide recovered (%)			
	0	3	10	30
ATP	100	75	57	10
ADP	7	18	45	90
AMP	2	2	3	7

kinase or other ADP-consuming activity under these conditions of low ATP concentration (Table 6).

Exchange of bound phosphate with ATP

Turnover of the P bound during incubation with [32 P]ATP was examined by adding non-radioactive MgATP to the 2.5ml. system at various times during the reaction, allowing a given period for further hydrolysis (usually 3 or 10sec.) and then denaturing the microsomes with trichloroacetic acid as usual. Because of the importance of ATP concentration in determining the time-course of bound P formation, and particularly the relative

size of the Na^+ -dependent increment, the observations were made at 20 and 200 μM -ATP concentrations. At both substrate concentrations the Na^+ -dependent increment in the bound P exchanged rapidly and completely (Fig. 6). This component accounted for the major part of the label at the lower ATP concentration, but at 200 μM -ATP it comprised less than 50% of the P bound during the first minute of the reaction by which time 50% of the ATP had been split; after 3min. when no ATP remained, but the bound P level had not declined, no exchangeable component could be demonstrated. In the absence of added Na^+ , a small exchangeable component was demonstrable over the first 90sec. of the reaction with 20 μM -ATP; initially this amounted to some 50% of the binding, but at 80sec. the proportion was considerably less. At the higher ATP concentration no exchangeable fraction could be demonstrated in the Na^+ -free system; in fact, in some experiments bound P appeared to increase on addition of unlabelled ATP during the first minute of the reaction.

Effect of ADP. This is shown in Table 7 for an experiment on the 2.5ml. scale. ADP equimolar with ATP was without effect on either the Na^+ -dependent binding or on the ATPase activity, but marked inhibition was seen at ATP:ADP ratio 1:10. This ratio is approximately the same as that reported by Post *et al.* (1965) for inhibition of binding and ATP hydrolysis in a kidney membrane preparation.

Effects of ouabain. The action of ouabain on the Na^+ -dependent binding in the 2.5ml. system varied with the concentration of sodium chloride (Table 8 and Fig. 7). At 15mm-sodium chloride, where ATPase activity was maximal but the binding submaximal, ouabain consistently stimulated binding while inhibiting the Na^+ -activated component of the ATPase. This effect was immediate, did not require preincubation and also occurred when the agent was added to the reaction mixture after 15sec. of incubation in its absence (ATP was 42% split at this point). Stability of the Na^+ -dependent bound P over the period 10–60sec. was not altered.

Fig. 6. Turnover of bound P. Ox-brain microsomes were incubated with [32 P]ATP at 37°, with or without 15mm- NaCl , in the 2.5ml. system; solvent treatment was not applied. Other procedural details were as described in the text. After a given interval indicated by the arrows, non-radioactive ATP, equal to eight times the initial amount of [32 P]ATP, was added in 0.2ml. and the reaction allowed to proceed for a further period. ●, Na^+ -dependent bound P; ○, Na^+ -dependent bound P after addition of non-radioactive ATP; ■, P bound in absence of Na^+ ; □, P bound in absence of Na^+ after addition of non-radioactive ATP. (a) Turnover with 20 μM -ATP. (b) Turnover with 200 μM -ATP.

Table 7. Effect of ADP on bound phosphate formation and ATP hydrolysis

Guinea-pig microsomes were incubated for 10sec. with [32 P]ATP (20 μM) on the 2.5ml. scale. Concentration of NaCl was 15mm; solvent treatment was not applied. ADP (sodium salt) was included in the reaction mixture at the concentrations indicated; additional Mg^{2+} was not added.

Concn. of ADP (μM)	0	20	200	1000
Bound P ($\mu\text{moles/mg. of protein}$)	21	20	12	6
ATP split (%)	33	36	17	7

Table 8. *Effect of ouabain on bound phosphate formation and ATP hydrolysis*

Ox-brain microsomes were incubated for 10sec. with [³²P]ATP (20 μM) at 37° on the 2.5 ml. scale; solvent treatment was not applied. Before the reaction the microsomes (in 0.2 ml. of sucrose medium) were incubated in 1.9 ml. with ouabain in the absence of buffer or cations at 37° for 5 min.; in control tubes preincubation was in deionized water. The required cations and tris buffer were then added in 0.5 ml., the mixture was stirred for 20 sec. and the reaction started with [³²P]ATP. Final concentration of ouabain was 0.1 mM. Results from two (or in one case three) independent experiments are given.

Na ⁺ (mm)	Ouabain	Bound P (μmoles/mg. of protein)	ATP split (%)
0	0	28, 22, 24	21, 30
0	present	32, 35, 40	23, 29
15	0	46, 38	47, 55
15	present	54, 55	28, 30
100	0	71, 71	34, 40
100	present	49, 50	15, 17

Fig. 7. *Effect of ouabain on time-course of bound P formation and ATP hydrolysis at 37° by guinea-pig microsomes, with 20 μM-ATP and in the presence of 15 mM-NaCl. The ouabain was incorporated in the basic medium to give final concentration 0.1 mM and the reaction was started as usual by addition of microsomes. Solvent treatment of the precipitates was not applied; other details were as described in the text. Time-courses of: ●, control reaction; ○, with 0.1 mM-ouabain.*

Ouabain also enhanced, to approximately the same extent, bound P formation in the absence of Na⁺, without affecting ATP hydrolysis. When the

reaction was carried out with 100mM-sodium chloride (conditions giving maximal binding with submaximal ATPase activity) ouabain reduced the level of bound P and further inhibited the ATPase. Preincubation was also not essential for this effect. This dual action of ouabain was unexpected and is discussed below.

Labelling of phosphorylserine. The radioactivity of phosphorylserine derived from microsomes incubated with 20 μM-ATP for 10sec. was zero in a guinea-pig preparation and very low in two different ox preparations (Table 9); the small amount of labelling found in the latter case was insensitive to Na⁺. As was expected, phosphorylserine labelling became apparent at higher ATP concentrations, but here also Na⁺ was without effect.

DISCUSSION

Complexity of binding reaction

From the effects of Na⁺ ions and ATP concentration on the time-course and other properties of the binding reaction in our microsomal preparation two types of bound phosphate can be distinguished: a relatively stable type produced in Mg²⁺-dependent reactions and an unstable type that requires Na⁺ in addition to Mg²⁺ in the reaction mixture. Except for the possibility of a quantitatively minor interrelation mentioned below it seems clear that the two types are independently produced by distinct microsomal enzymes. As suggested by Rodnight & Lavin (1966) the stable fraction probably results from several different phosphorylating reactions involving as yet unidentified acceptors; the rates of these reactions are comparatively slow and are responsive to increasing ATP concentration. Addition of Na⁺ to the reaction mixture can now be seen to divert part or all of the ATP to a very rapid binding system which yields the unstable type of bound P. In this case it appears likely that only one reaction is involved. Its characteristics are clearly seen in the present study only at ATP concentration 20 μM, where virtually all of the bound P appears to be of the Na⁺-dependent type, as is shown by its decline to near zero-time levels as ATP hydrolysis approaches completion and by the observation that the bound P almost completely exchanges with non-radioactive ATP during the reaction. At higher substrate concentrations, on the other hand, an increasing proportion of the ATP is apparently utilized by the other reactions and therefore only part of the phosphate bound in the presence of Na⁺ is unstable or exchangeable. This competition for ATP between two distinct types of binding reaction would appear to explain why the effect of Na⁺ on the level of total bound P depends both on time of incubation and on substrate concentration.

Table 9. *Lack of effect of Na⁺ on labelling of phosphorylserine at low ATP concentrations*

Microsomes were labelled at 37° in the 2.5 ml. system for 10 sec. under the conditions indicated in the Table; the denatured precipitates were washed with acid and solvents by the procedure given in the text. From each sample a portion was taken for determination of total bound P and the remainder (equivalent to 3 mg. of microsomal protein) was hydrolysed for 6 hr. at 100° with 2N-HCl. Total counts in the free phosphorylserine released from this amount of protein were determined after electrophoresis (see Rodnight & Lavin, 1966) by scanning the paper at 5 mm. intervals with a Geiger counter.

Sources of microsomes	Concn. of Mg-[³² P]ATP (μ M)	Bound P (μ moles/mg. of protein)		Counts/min. in phosphorylserine	
		Na ⁺ absent	15 mM-NaCl	Na ⁺ absent	15 mM-NaCl
Ox brain (H10)	20	9	28	245	260
Ox brain (H11)	20	14	27	130	140
			100 mM-NaCl		100 mM-NaCl
Guinea-pig brain	20	9	29	0	0
	200	84	93	300	280
	500	180	170	500	460

According to this view the features of the Na⁺-dependent reaction that contribute to this dual effect are its very rapid rate and the fact that the level of bound P produced in the reaction is limited by turnover. The relation to substrate concentration appears to arise because, as mentioned above, the rates of the reactions producing stable bound P are sensitive to increasing ATP levels over the concentration range employed, whereas in the Na⁺-dependent reaction the rate is too rapid to measure even at the lowest ATP concentration. Thus at 1 mM-ATP the rate of the former approaches that of the latter and the interval before the two time-courses cross is correspondingly short.

We are inclined to consider the exchangeable component of the binding observed at 20 μ M-ATP in the absence of Na⁺ as reflecting activity of the Na⁺-dependent type, possibly activated by bound Na⁺, rather than a distinct enzyme system producing another type of bound P exhibiting turnover. Further fractionation of the preparation might settle this point.

Relation of the binding to ATPase activity

The Na⁺-dependent binding appears to correspond to that described in other membrane preparations from brain by Skou (1965), Gibbs *et al.* (1965), Hokin *et al.* (1965) and Nagano *et al.* (1965), in an *Electrophorus* preparation by Albers *et al.* (1963) and in a kidney preparation by Post *et al.* (1965). For reasons discussed below the relation of our work to the binding system described by Ahmed & Judah (1965) is less clear.

In all these systems, and equally in the present work, the Na⁺-dependent binding shows many features which suggest its participation in the

(Na⁺,K⁺)-ATPase reaction according to the following theoretical schemes:

where E is an enzyme binding ATP and X is a non-enzymic structure cyclically phosphorylated and dephosphorylated by distinct enzymes, and with Na⁺ required for the phosphorylation step and K⁺ for the hydrolysis of the intermediate in (3) or (5). In advancing this interpretation several groups of workers (see especially Post *et al.* 1965; Gibbs *et al.* 1965) have equated the bound P in their experiments with a phosphorylated ATPase enzyme. We would agree that the evidence that the Na⁺-dependent binding is related to the major ATPase activity of the preparation is indeed very strong; in the present work this conclusion is consistent with the general pattern of sensitivity of the binding to cations and to ouabain, its very rapid initial rate and the formation of a plateau in its time-course at 37°, the dependence of this plateau on ATP levels and the fact that the bound P exchanges rapidly with ATP added during the course of the reaction. It is therefore very unlikely that the binding and (Na⁺,K⁺)-ATPase activities are independent reactions. On the other hand it appears premature to identify the bound P with the hypothetical E~P or X~P in the absence of evidence excluding other interpretations. Amongst these is the possibility that it is a secondary product derived from E~P or X~P by a transphosphorylation occurring either during incubation as a side reaction or by migration from E or X during acid

denaturation of the enzyme. In the present heterogeneous system migration could possibly occur either within the ATPase enzyme (analogous to the suspected migration of phosphate in dialkyl-phosphate labelled esterases) or to suitably positioned acceptors in an associated molecule. Weighing against these reservations are the results of Nagano *et al.* (1965), who detected a small amount of Na⁺-dependent bound P in their preparation after stopping the reaction at neutral pH with a detergent and separating the labelled macromolecules by gel filtration. The amount detected was only 30% of that found after acid denaturation. If this 'neutral' bound P can be shown to involve the same bond, migration during acid denaturation is excluded.

A secondary phosphorylation of adjacent groups during the reaction as a source of bound P would seem less likely if it could be shown that the initial rate of bound P formation equalled or exceeded that of ATP hydrolysis. Here it may be noted that the possibility of demonstrating an adequate binding rate depends on a quantitative recovery of bound P through the acid-denaturation and washing procedures, and that a positive finding would still leave open the question of migration, supposing this also occurred without loss of P. We have been unable to obtain any clear evidence on these points. Comparing the total binding rate at 0° in the presence of 100 mM-sodium chloride over the first 5 sec. (Fig. 3) with the maximum ATPase rate exhibited by the preparation, it can be seen that the latter is proceeding at least 20 times as fast. This comparison, however, ignores the possibility that ATP hydrolysis may be proceeding by more than one route, even in the presence of Na⁺ and K⁺. It would be attractive to compare, as Ahmed & Judah (1965) have done in their system at 6°, the Na⁺-dependent increment in the binding with the (Na⁺, K⁺)-dependent increment in hydrolysis, but this procedure assumes that the correction made from ATPase activity not requiring univalent cations is a valid one; this assumption seems particularly unlikely in our low substrate system where only a limited quantity of ATP is available. In the event we could find no (Na⁺, K⁺)-increment in ATPase activity at 0°, and at higher temperatures where a measurable increment occurred, the initial rate of binding was too rapid to determine with the conventional equipment at our disposal.

Another rather different interpretation of the origin of the bound P does not require a phosphorylated intermediate in the ATPase mechanism. Although we have shown that the bound P as measured cannot be in the form of ATP, it is conceivable that a transfer of phosphate from ATP bound in the enzyme-substrate complex to strategically situated acceptors in the membrane structure

might occur on acid denaturation. The reaction sequence would then be:

but on acid denaturation:

with Na⁺ presumably involved in (1), K⁺ in (6) and where Y-P is the bound P recovered in the denatured microsomes. We consider such a formulation unlikely, however, on the grounds that inhibition by ADP of the binding and ATPase reactions can be more readily explained by a mechanism involving a phosphorylated intermediate.

The view that ADP inhibits ATPase activity by altering the balance of reactions (2) or (4) implies that under suitable conditions the enzyme system would exhibit ADP-ATP exchange activity. The position here is that more than 96% of the ADP-ATP exchange activity of brain microsomes has now been shown by Stahl, Sattin & McIlwain (1966) to have no direct relation to the ATPase system; the status of the remaining 3-4%, which was not separated, remains uncertain, although part of it was attributable to adenylate kinase activity. The present observations would tend to suggest that the residual exchange activity may indeed reflect the ATPase system, and, as Stahl *et al.* (1966) have pointed out, demonstrable exchange activity may be very low under conditions where E ~ P or X ~ P is being rapidly removed by (3) or (5). There is considerable indirect evidence for the occurrence of a phosphorylated intermediate in the course of myosin ATPase action (Yount & Koshland, 1963; Imamura, Kanazawa, Tada & Tonomura, 1965), although this enzyme does not exhibit ADP-ATP exchange activity (Koshland, Budenstein & Kowalsky, 1954). Product inhibition, particularly by ADP but also by P_i, of myofibrillar ATPase has been described (Bendall, 1961). Inhibition by P_i of the present system could not be demonstrated: when the Na⁺-dependent binding and ATPase time-courses were compared in tris and phosphate buffer they were found to be similar (R. Rodnight, unpublished work).

Effects of ouabain and cations

Accepting that the Na⁺-dependent bound P is either identical or closely related to the E ~ P of (2) or X ~ P of (4) certain of the cation effects require further comment. With regard to the action of K⁺ it has been suggested by Skou (1965) that the microsomal ATPase may proceed by two routes, one involving a phosphorylated intermediate and not requiring K⁺ at any stage, and the other (the normal route) depending on K⁺ as well as Na⁺ and

proceeding by a route analogous to reaction (6) above. According to this interpretation therefore the action of K^+ in reducing the steady level of bound P is not one of accelerating (3) or (5) but of diverting ATP to the non-phosphorylating route. Part of the evidence quoted for this suggestion was an observation that under certain conditions the reduction in bound P levels by K^+ was not accompanied by an increase in ATP hydrolysis. We prefer to interpret the same findings in the present work (see data in Fig. 5 for 15 mM-sodium chloride) in terms of a phosphoryl intermediate by assuming that at very low ATP concentration the acceleration in turnover given by K^+ under these conditions is just balanced by the reduction in the number of sites reacting; this would imply that (2) or (4) is rate-limiting, especially in these circumstances. At K^+ concentrations greater than 1 mM, where both binding and ATPase are inhibited in parallel, K^+ may be competing with Na^+ in (2) or (4), as well as accelerating (3) or (5).

Stimulation of Na^+ -dependent bound P formation by ouabain in the absence of added K^+ has not been previously reported; in the other experimental systems referred to above (see especially Post *et al.* 1965; Gibbs *et al.* 1965; Skou, 1965) the observed effect has been one of reducing the level of bound P with only Na^+ present and of increasing it to about the same level in the presence of added K^+ . A possible explanation of the stimulation observed in the present work is that it is due to an interaction of ouabain with the intrinsic K^+ in our preparation; this would be consistent with the conclusion already reached that the residual K^+ is available to the enzyme system. At the suboptimum Na^+ concentration therefore ouabain appears to displace or inactivate bound K^+ and thus allow the level of $E \sim P$ or $X \sim P$ to build up. At the higher Na^+ concentration ouabain seems to act primarily at reactions (2) or (4), rather than (3) or (5) where we earlier postulated under these conditions displacement of the traces of bound K^+ by Na^+ as well as by ouabain; consistent with this suggestion is the observation that ouabain at 100 mM-sodium chloride further inhibits the already partially inhibited ATPase (Table 8). Probably some inhibition of (2) or (4) also occurs at the lower Na^+ concentration of 15 mM, since the ouabain-induced increase in bound P level apparently falls short of that reached with 100 mM-sodium chloride; in this connexion Post *et al.* (1965) found that inhibition of binding by ouabain in their kidney-membrane preparation was relatively small at suboptimum Na^+ concentrations. The small stimulation by ouabain of our binding occurring in the absence of added univalent cations confirms the impression that a fraction of the binding observed under these conditions has the properties of the Na^+ -dependent reaction; however,

the lack of any effect on the ATPase activity under these conditions suggests that the (Na^+, K^+) -component is relatively small.

Phosphorylserine component of the bound P

The absence of a phosphorylserine component in our Na^+ -dependent bound P appears to differentiate it from the bound P described by Ahmed & Judah (1965): here Na^+ apparently stimulated incorporation of ^{32}P into phosphorylserine residues isolated from a partially hydrolysed preparation by a technique similar to that used in the present work. Other experimental conditions employed were, however, rather different from ours; in particular their membrane preparation had been treated with deoxycholate and incubation was with 5 mM-ATP at 6°. The proportion of the total bound P linked to serine in the experiments of Ahmed & Judah (1965) is indeterminate since the method involved an uncertain loss of phosphorylserine during hydrolysis. It is possible therefore that the phosphorylserine component only comprises a minor fraction of their total bound P and that the remainder consists of the unstable type described here; labelled phosphorylserine might then arise from a side reaction of the major fraction (or its derivatives) with adjacent serine groups, either during the reaction or as a result of acid-denaturation.

The same considerations also apply to the bound P that turns over more rapidly when electrical pulses are applied to slices of cerebral cortex *in vitro*, as this also contains a phosphorylserine component (Trevor & Rodnight, 1965). At first sight this feature would again appear to distinguish it from the bound P described in the present work, but here also it is conceivable that the labelled phosphorylserine is a relatively stable secondary product derived from an unstable intermediate involved in membrane ATPase action.

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