Enzyme Transfer of Phosphate from Adenosine Triphosphate to Protein-Bound Serine Residues in Cerebral Microsomes

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1. Microsomes from guinea-pig brain grey matter were incubated with [32 P]ATP at 3mM concentration and the phosphate bound to the acid-washed, lipid-free residue was determined. 2. The binding process was Mg^{2+} -dependent and resulted in the transfer of about 1–2 μ moles of phosphate/mg. of protein/min. Under the conditions used univalent cations (Na^+ , K^+ and Li^+) inhibited the binding. 3. An unspecified proportion of this bound phosphate could be recovered in protein-derived phosphorylserine. The yield of labelled phosphorylserine was also decreased by univalent cations. 4. The bound phosphate formed with 3mM-MgATP was stable; addition of Na^+ or K^+ ions to the already labelled preparation had no effect on the bound phosphate level. 5. Bound phosphate was also formed when a solubilized fraction of the microsomes was incubated with ATP; univalent cations also inhibited this process. 6. *p*-Chloromercuribenzoate reduced the binding by about 25%; the inhibition was restored by cysteine.

Protein-bound phosphorylserine groups, located in the microsomal fraction of guinea-pig cerebral cortex, turn over faster *in vitro* when electrical pulses are applied to slices of the intact tissue by the technique of McIlwain (McIlwain & Rodnight, 1962; Trevor & Rodnight, 1965). The microsomal fraction used in these studies is believed to contain fragments of the plasma membranes of cortical cells. The increased turnover of phosphoprotein presumably represents a faster rate of transfer to membrane proteins of phosphate derived from intracellular nucleotides, of which ATP is the major component, though not necessarily the immediate phosphate donor. Although neither the identity nor the function of the sensitive protein is known, a possible explanation of the augmented transfer is that it reflects the participation of a membrane phosphoprotein in some aspect of the utilization of the bond energy of ATP for cation transport. This interpretation is consistent with recent observations clearly establishing that electrical pulses induce major changes in cation flux across cell membranes in the tissue (Keesey & Wallgren, 1965; Keesey, Wallgren & McIlwain, 1965), and is also supported by earlier experiments in which it was found that the response of the phosphoprotein to electrical pulses was greatly reduced by the inclusion of ouabain or the substitution of Na^+ by Li^+ in the incubation

medium (Ahmed, Judah & Wallgren, 1963; Trevor & Rodnight, 1965).

The purpose of the work reported in the present and following two papers has been to explore with isotopic methods the transfer of phosphate from ATP to structures in the isolated microsomal fraction with the aim of creating the basis for the further investigations of the findings in the intact tissue. The enzymic mechanisms involved in these transfer processes are only poorly understood. Although it appears likely (Kothary, 1964) that a considerable number of membrane proteins in the microsomal fraction can accept phosphate from ATP through the mediation of kinase enzymes, none has been isolated or characterized from this source. A kinase phosphorylating an extrinsic substrate, the egg-yolk protein phosvitin, occurs in the microsomal fraction of brain tissue and is stimulated by univalent cations (Rodnight & Lavin, 1964); it presumably possesses a native substrate in the membrane. However, the maximum rate of transfer of phosphate to phosvitin catalysed by this enzyme is very low, and on this and other grounds it was considered unlikely to be involved in cation transport (Decsi & Rodnight, 1965). Possibly of greater relevance are the observations of several different workers (for references see Rodnight, Hems & Lavin, 1966) pointing to the involvement of a

phosphorylated protein, or group closely associated with protein, in the mechanism of action of the $(\text{Na}^+, \text{K}^+)\text{-ATPase}^*$, now widely held to represent in isolated membrane fragments the mechanism for cation transport (McIlwain, 1963; Judah & Ahmed, 1964; Skou, 1965).

The present paper is mainly concerned with features of the binding found in the presence of 3mM-ATP, a concentration approximating to the mean intracellular concentration of this nucleotide in the intact tissue (McIlwain, 1966). In the following papers (Rodnight *et al.* 1966; Hems & Rodnight, 1966) results are described with much lower ATP concentrations, where a radically different type of binding became apparent.

EXPERIMENTAL

Preparation of microsomes. A microsomal fraction rich in membrane fragments was prepared from adult guinea-pig brain as follows. A typical preparation utilized six adult guinea pigs. The brains were rapidly removed and cooled by immersion in ice-cold 0.32M-sucrose (containing 0.5mM-EDTA adjusted to pH 7 with tris). Cerebral cortex was freed from white matter by careful dissection and dispersed in 10 vol. of the same sucrose medium in a glass tissue grinder fitted with a Teflon pestle (clearance 0.15mm. on the diameter of the tube). The dispersion involved 10–12 excursions of the pestle revolving at approx. 2000 rev./min.

A low-speed fraction was first separated by centrifuging the dispersion at 6000 g-min. and the pellet was washed once by resuspension in half the original volume of medium used. A mitochondrial fraction was then deposited from the combined supernatants by centrifuging in the MSE 25000 centrifuge at 1.2×10^5 g-min. The mitochondrial pellet was washed twice with a volume of medium equal to half that of the original post-nuclear material. To obtain the microsomes the combined post-mitochondrial supernatants were centrifuged at 2.34×10^6 g-min. in the MSE 25000 machine. The supernatant was discarded and the microsomal pellet resuspended by gentle dispersion in sucrose medium to give an approximate concentration of 5mg. of microsomal protein/ml. Finally this suspension was centrifuged at the speed (1.2×10^5 g-min.) used for mitochondrial separation to deposit any contaminating mitochondria.

The yield of microsomal protein in 19 preparations ranged from 12 to 20 mg./g. of cortex (mean 16.8 mg.). This variability largely appeared to depend on the amount of material discarded after the final spin, where separation was often ill-defined. The microsomal suspensions were stored in sucrose solution (containing 0.5mM-EDTA at pH 7) at -20° ; no loss of ATPase or binding activity was observed for periods at least up to 8 weeks. The preparations were always tested for $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity in a medium containing 3mM-MgATP, 30mM-tris-HCl (pH 7.5), 100mM-NaCl and 30mM-KCl. Specific activities under these conditions were in the range 48–83 $\mu\text{moles of P/mg. of protein/hr.}$; without Na^+ or K^+ present the ATPase activity lay in the range 15–28 $\mu\text{moles of P/mg. of protein/hr.}$

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

Preparation of $[\text{}^{32}\text{P}]\text{ATP}$. The $\beta\gamma\text{-}^{32}\text{P}$ -labelled ATP used in most experiments was prepared with rat-liver mitochondria by the procedures described earlier (Rodnight & Lavin, 1964). In a few experiments $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of higher specific radioactivity was used and was prepared by a modification of the method of Pfeleiderer (1961) kindly communicated by Mr C. Hilberg of the Institute of Physiology, University of Aarhus, Denmark. Carrier-free $[\text{}^{32}\text{P}]\text{P}_i$ was obtained from The Radiochemical Centre, Amersham, Bucks., and after neutralization was used without further treatment. The sodium salt of ADP came from Sigma Chemical Co., St Louis, Mo., U.S.A. All other special reagents, including the enzymes (for which the catalogue numbers are given below), were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany.

Fructose 1,6-diphosphate (24mg., sodium salt) dissolved in 30ml. of 50mM-tris-HCl (pH 7.4) was allowed to react with 200 $\mu\text{l.}$ of aldolase (ALD 15419) for 10min. at room temperature. The resulting solution of triose phosphates was then poured into another flask containing, in a volume of approx. 1 ml., $[\text{}^{32}\text{P}]\text{P}_i$ (5mc), NAD (9 μmoles), MgCl_2 (120 μmoles) and ADP (15 μmoles). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH 15146, 100 $\mu\text{l.}$) and 3-phosphoglycerate kinase (PGK 15745, 50 $\mu\text{l.}$) were then added and the mixture stood for 25min. The reaction was stopped by holding the flask in a boiling-water bath for 2 min.; after cooling, carrier ATP (disodium salt, 5 μmoles) was added and the solution filtered on to a column of Dowex 1 (X10; 200–400 mesh; formate form). The chromatographic procedures for the purification and isolation of the tris salt of the $[\text{}^{32}\text{P}]\text{ATP}$ by charcoal adsorption etc., were as given by Rodnight & Lavin (1964).

With this method, $[\text{}^{32}\text{P}]\text{ATP}$ with a specific radioactivity 0.1–0.4 mc/ μmole of $\gamma\text{-P}$ was regularly obtained. The P_i contamination was negligible and the radioactivity of the P_i never accounted for more than 2% of the total radioactivity.

Reaction system and general procedures. Basically the reaction system contained tris-HCl buffer (pH 7.5, 30mM), the tris salt of $[\text{}^{32}\text{P}]\text{ATP}$ (3mM; $1\text{--}2 \times 10^5$ counts/min./ μmole of acid-labile P or $\gamma\text{-P}$), MgCl_2 (3mM), microsomal protein (1mg.) and other additions as stated, to a final volume of 1ml. The reaction was started by the addition of microsomes and stopped with 1.5ml. of ice-cold 10% (w/v) trichloroacetic acid. The cooled tubes were centrifuged and portions of the supernatants taken for P_i determination by a colorimetric method. The precipitates were washed in the centrifuge three times with 5ml. of ice-cold 10% (w/v) trichloroacetic acid, once with 1ml. of ethanol, once with 2ml. of chloroform-methanol (2:1) and finally with 1ml. of ethanol; for each wash the precipitate was dispersed with a loosely fitting glass pestle. The treated protein was then dissolved in 1ml. of N-NaOH, diluted to 10ml. with water, and total counts were determined in a M6H liquid counter (20th Century Electronics Ltd., counting efficiency about 10%). Controls consisting of the complete reaction medium to which trichloroacetic acid was added before the microsomes, were always carried out in parallel.

Tris-adenosine triphosphate. The sodium salt of ATP (Sigma Chemical Co.) was passed through a column of Dowex 50 (X12) and the effluent brought to pH 7 with m-tris. The concentration of the solution was adjusted by measuring E_{260} .

Determination of ATPase. Inorganic phosphate in the

trichloroacetic acid supernatants was determined colorimetrically by a micro-modification of the method of Martin & Doty (1949), omitting the silico-tungstate reagent.

Protein. This was determined either according to Lowry, Rosebrough, Farr & Randall (1951) or by the biuret method of Gornall, Bardawill & David (1949), with crystallized bovine plasma albumin as standard.

Radioactive phosphorylserine. An electrophoretic method was used in addition to the ion-exchange method previously employed (Trevor & Rodnight, 1965). Both methods utilized the same procedures for preparing partial acid hydrolysates of microsomal protein. In the electrophoretic method these were applied to Whatman no. 3MM paper and subjected to 15 v/cm. for 5 hr. at room temperature in 50 mm-pyridine acetate buffer, pH 3.5. Each determination used a volume of hydrolysate equivalent to 3 mg. of protein applied to the paper in the form of a spot. Total counts in the phosphorylserine spot were determined by scanning the paper with a Geiger-Müller tube at 5 mm. intervals (for further details see Hems & Rodnight, 1966). Separation of phosphorylserine from P_i was good; addition of carrier phosphorylserine (0.5 μ mole) assisted location of the radioactive phosphorylserine on the paper with ninhydrin, but was not essential as the radioactive peaks were similar in shape and magnitude whether carrier was added or not.

RESULTS

General features of binding reaction. In exploratory experiments transfer of phosphate to microsomal material was examined by the ATPase assay of the system of Schwartz, Bachelard & McIlwain (1962), with 3 mm-ATP and a concentration of microsomal protein of 1 mg./ml. Use of the high concentration of protein was necessary to obtain sufficient material for a satisfactory determination of bound ^{32}P . Under these conditions and with Mg^{2+} , Na^+ and K^+ present, the γ -phosphate of the ATP was completely hydrolysed in 7 min.; by 12 min. a further 0.8 μ mole of P_i had been produced, presumably due to some adenylate kinase activity in the preparation (Fig. 1). Concurrently with the ATP hydrolysis there occurred a time-dependent binding of phosphate by the microsomes in a form stable to treatment with acid and chloroform-methanol; this process was inhibited by univalent cations (Table 1 and Fig. 1). No significant binding occurred in the absence of Mg^{2+} but experiments with pre-hydrolysed [^{32}P]ATP showed that about 10% of the label probably represents physical adsorption of ^{32}P released by the ATPase during the course of the reaction.

The initial rate of the binding was approx. 0.1% of that of the concomitant ATPase reaction (Fig. 1)

Table 1. Effect of cations on phosphate binding

The reaction mixture (final vol. 1 ml.) contained tris-HCl buffer (pH 7.5, 30 mm), the tris salt of [^{32}P]ATP (3 mm; $\beta\gamma$ -labelled with 1.2×10^5 counts/min./ μ mole of acid-labile P) and the chlorides of the various cations as stated. The reaction was started by adding 1 mg. of microsomal protein and stopped after 2 min. at 37° with 1.5 ml. of cold 10% trichloroacetic acid. Zero-time controls (for which all results are corrected) were obtained by adding the acid to the reaction mixture before the microsomes. The precipitated protein was washed, treated with solvents and counted as described in the text. Results are the mean of duplicate assays.

Expt. no.	Cations present (mm)	Phosphate binding (m μ moles/mg. of protein)	ATPase (μ moles of P/mg. of protein)
1	Mg ²⁺ , 3	1.75	—
	Mg ²⁺ , 9	2.62	—
	Mg ²⁺ , 16	2.02	—
2	Mg ²⁺ , 3	2.00	1.73
	Mg ²⁺ , 3; Ca ²⁺ , 2	1.49	1.77
	Mg ²⁺ , 0; Ca ²⁺ , 3	0.12	1.40
3	Mg ²⁺ , 3	1.30	—
	Mg ²⁺ , 3; K ⁺ , 20	1.04	—
	Mg ²⁺ , 3; K ⁺ , 50	0.76	—
4	Mg ²⁺ , 9	2.27	1.35
	Mg ²⁺ , 9; K ⁺ , 300	0.05	1.18
	Mg ²⁺ , 9; Na ⁺ , 50	0.93	1.29
	Mg ²⁺ , 9; Na ⁺ , 150	0.76	1.35
5	Mg ²⁺ , 9	2.68	1.19
	Mg ²⁺ , 9; Na ⁺ , 200	0.47	1.13
	Mg ²⁺ , 9; Li ⁺ , 200	0.49	1.09

Fig. 1. Time-course of bound P formation and ATP hydrolysis in guinea-pig microsomes with 3 mm-MgATP. Experimental details were essentially the same as given in Table 1, except that [γ - ^{32}P]ATP was used. ●, Only Mg^{2+} present; ○, 100 mm-NaCl and 30 mm-KCl present.

and the quantity of bound phosphate was therefore very small compared with that released to the medium. Over 90% of the bound phosphate was released as P_i by incubation with mild alkali for 18 hr. (Table 2), suggesting incorporation into protein phosphorylserine groups, although phosphate bound in certain other linkages might also be released by this treatment, which completely dissolved the protein. However, determination of protein phosphorylserine radioactivity (Table 3) showed that part, at least, of the bound phosphate was recoverable as phosphorylserine, although the exact proportion could not be assessed since the methods used yielded only an indeterminate fraction of the total protein phosphorylserine in the sample. The phosphorylserine labelling was inhibited by univalent cations.

Table 2. *Lability of bound phosphate to mild alkali treatment*

The reaction was carried out as described in Table 1. After washing with trichloroacetic acid and removal of lipids the precipitate was either reserved for determination of total counts or incubated at 37° for 18 hr. with 0.2 ml. of $N-NaOH$. The incubated samples were neutralized with $HClO_4$ and the P_i was extracted with isobutanol-benzene mixture after conversion into phosphomolybdic acid.

Cations present (mm)	Time of incubation (min.)	Counts in untreated precipitate	Counts in isobutanol-benzene extract
Mg^{2+} , 9	0	121	38
Mg^{2+} , 9	2	519, 572	425, 424
Mg^{2+} , 9; Na^+ , 200	2	320, 316	233, 211

The rate of phosphate binding was apparently higher with $[\beta\gamma\text{-}^{32}P]ATP$ than with the γ -labelled product. This is evident by comparing the values for binding shown in Tables 1-6, where $\beta\gamma$ -labelled ATP was used, with those in Fig. 1 where the precursor was γ -labelled ATP; other unpublished experiments with $[\gamma\text{-}^{32}P]ATP$ have consistently confirmed this difference. The difference is too great to be entirely due to adenylate kinase activity and is probably a consequence of inaccuracies in measuring the specific radioactivity of the γ -phosphate of the doubly labelled preparation. This was obtained by isolating and determining the acid-labile phosphate and assuming that the label was equally distributed between the γ - and β -positions; if, in fact, the distribution tended to be in favour of the γ -position, then the binding rate would have been apparently high.

In two experiments the specific radioactivity of the alkali-labile bound phosphate and the inorganic phosphate liberated (both extracted as phosphomolybdic acid and determined by reduction to molybdenum blue) were compared with that of the $[^{32}P]ATP$ (specific radioactivity 3×10^5 counts/min./ μ mole of γ -P) at several time-intervals from 13 sec. to 10 min. The specific radioactivity of the bound $[^{32}P]$ phosphate was never more than 10% of that of the $[^{32}P]ATP$ or of the inorganic $[^{32}P]$ -phosphate in the medium. The radioactivity of the latter was maximal and close to that of the $[^{32}P]$ -ATP at 13 sec., but thereafter slowly declined, presumably due to a gradual hydrolysis of the β -phosphate of ATP by adenylate kinase.

Effects of cations. Data are given in Table 1 on the effect of Mg^{2+} and Ca^{2+} on the binding reaction. The latter inhibited the binding in the presence of

Table 3. *Labelling of protein phosphorylserine groups in cerebral microsomes*

The reaction mixture contained in 10 ml. 500 μ moles of tris-HCl (pH 7.6) and labelled precursors and cations as indicated. Microsomal protein (10 mg.) was added at zero time; temperature was 37°. In Expt. A $[^{32}P]$ -phosphorylserine was determined as described by Trevor & Rodnight (1965), in Expt. B by the electrophoretic method given in the Experimental section.

Precursor (mm)	Cations (mm)	Time of incubation (min.)	Radioactivity of $[^{32}P]$ phosphorylserine (counts/min./ μ mole of P)
Expt. A			
$[\beta\gamma\text{-}^{32}P]ATP$, 1	Mg^{2+} , 3	0	120
$[\beta\gamma\text{-}^{32}P]ATP$, 1	Mg^{2+} , 3	5	960
$[\beta\gamma\text{-}^{32}P]ATP$, 1	Mg^{2+} , 3; Na^+ , 200	5	545
$[^{32}P]Na_2HPO_4$, 1	Mg^{2+} , 3	5	40
Expt. B			
$[\gamma\text{-}^{32}P]ATP$, 3	Mg^{2+} , 3	10	955
$[\gamma\text{-}^{32}P]ATP$, 3	Mg^{2+} , 3; Na^+ , 200	10	505
$[\gamma\text{-}^{32}P]ATP$, 3	Mg^{2+} , 3; K^+ , 200	10	330
$[\gamma\text{-}^{32}P]ATP$, 3	Mg^{2+} , 3; Li^+ , 200	10	270

Total counts in phosphorylserine spot

Table 4. *Addition of univalent cations to ^{32}P -labelled microsomes*

The reaction mixture and general procedure were the same as given in Table 1. The additions of Na^+ and K^+ to the labelled system at 5 min. were made in a volume of 0.1 ml.

Cations present at zero time (mM)	Cation added at 5 min.	Phosphate binding ($\mu\text{moles/mg. of protein}$)			
		5 min.	8 min.	9 min.	15 min.
Mg^{2+} , 9	None	3.1	—	3.0	—
Mg^{2+} , 9	K^+ to 200 mM	—	—	3.2	3.0
Mg^{2+} , 9; K^+ , 200	None	1.4	—	—	—
Mg^{2+} , 9	None	2.5	2.8	—	—
Mg^{2+} , 9	Na^+ to 200 mM	—	2.9	—	—
Mg^{2+} , 9; Na^+ , 200	None	0.8	—	—	—

Table 5. *Effect of Na^+ and K^+ on phosphate binding in a soluble protein fraction*

A suspension of microsomes in tris buffer, pH 7, and vigorously stirred at 2° was brought to pH 10.5 with 0.1 N-NaOH and then immediately readjusted to pH 7 with 0.1 N-acetic acid. A portion of the treated suspension was reserved and the remainder centrifuged at $3 \times 10^6 \text{ g. min.}$ Proteins in the supernatant were concentrated by ultrafiltration dialysis under negative pressure with tris buffer outside the dialysis tubing. The sediment of insoluble proteins was resuspended in the same tris buffer. Binding and ATPase activities were determined by the procedure of Table 1.

Sample	Univalent cation (200 mM)	Phosphate bound ($\mu\text{moles/mg. of protein}$)	ATPase ($\mu\text{moles of P/mg. of protein}$)
Treated suspension	None	2.24	1.42
before centrifuging	Na^+	0.93	1.65
(100 mg.)	K^+	1.07	1.48
Insoluble fraction	None	1.48	1.25
(88 mg.)	Na^+	0.80	1.48
Soluble fraction	None	3.10	0.05
(12 mg.)	Na^+	0.95	0.02
	K^+	1.17	0.02

Mg^{2+} and showed very little activating effect on its own. Mg^{2+} stimulated the reaction maximally between 9 and 15 mM concentration; higher concentrations tended to reduce the activity, though not markedly.

The nature of the inhibition of the binding given by Na^+ and K^+ was first investigated by labelling a preparation in the presence of Mg^{2+} for 5 min. and observing the stability of the label after addition of Na^+ or K^+ to the reaction mixture. Since no loss of label occurred when incubation was continued for a further 3–10 min. (Table 4), it is clear that the univalent cations do not inhibit the binding by decreasing the stability of the bound phosphate. The stable nature of the phosphate bound in the reaction requiring only Mg^{2+} (see also Rodnight *et al.* 1966) together with the low initial rate of the binding reaction compared with the overall rate of P_i liberation (both with and without Na^+ and K^+) appear to exclude any involvement of the bound phosphate measured under these conditions in the ATPase activities of the preparation.

One explanation of the inhibition of the stable binding by Na^+ is provided in the following paper (Rodnight *et al.* 1966), where it is shown that Na^+ activates a reaction forming a labile type of bound phosphate that turns over rapidly; this reaction involves relatively few sites and is apparently related to the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity of the preparation. It seems, however, that other factors may be concerned in the inhibition. Thus K^+ and Li^+ , which do not activate the reaction forming labile bound phosphate, also inhibit the stable binding reaction (Table 1). This non-specific feature of the inhibition first suggested to us that the univalent cations were altering the degree of dispersion of the microsomal suspension in a manner that masked phosphate acceptor groups within the protein, an interpretation supported by an earlier finding that cerebral microsomes are more readily sedimented from dispersions of brain if the sucrose solution contains small amounts of univalent cations (Trevor & Rodnight, 1965). Other experiments, however, have tended to discount an

Table 6. *Effect of ouabain, thiol reagents and chlorpromazine on phosphate binding*

The reaction mixture and general procedure were the same as given in Table 1. The various agents were incorporated into the medium before starting the reaction by adding the microsomal protein.

Expt. no.	Cations present in medium (mm)	Agent (mm)	Phosphate binding (μ moles/mg. of protein)	ATPase (μ moles of P/mg. of protein)
1	Mg ²⁺ , 9	None	1.48, 1.40	—
	Mg ²⁺ , 9; Na ⁺ , 200	None	0.83, 0.94, 0.86	—
	Mg ²⁺ , 9; Na ⁺ , 200	Ouabain, 0.13	0.99, 1.05, 0.88	—
2	Mg ²⁺ , 9	None	3.07	—
	Mg ²⁺ , 9	<i>p</i> -Chloromercuribenzoate, 0.01	2.38, 2.12	—
	Mg ²⁺ , 9	<i>p</i> -Chloromercuribenzoate, 0.01, cysteine, 0.1	2.83, 3.22	—
	Mg ²⁺ , 9	Cysteine, 0.1	3.25, 3.26	—
3	Mg ²⁺ , 3	None	1.64, 1.54	0.41, 0.53
	Mg ²⁺ , 3	Chlorpromazine, 0.2	2.76, 2.66	0.27, 0.28
	Mg ²⁺ , 3; Na ⁺ , 200; K ⁺ , 30	None	0.91, 0.80	1.15, 1.17
	Mg ²⁺ , 3; Na ⁺ , 200; K ⁺ , 30	Chlorpromazine, 0.2	1.80, 1.82	0.27, 0.27

explanation of the inhibition in these terms. Of particular significance is the observation that Na⁺ and K⁺ at 200mm concentration inhibited the binding reaction in a soluble protein fraction of the microsomes without exerting any visible effect on the protein solubility (Table 5). The soluble fraction, comprising about 12% of the total microsomal proteins, was prepared by exposing a suspension of microsomes to pH 10.5 for a few seconds, readjusting the pH to 7.0 and centrifuging at 3×10^6 g.-min. (Kothary, 1964; Decsi & Rodnight, 1965). In the experiment of Table 5 approx. 80% of both the binding reaction and ATPase activity were recovered; of the binding reaction 18% was found in the soluble and 58% in the insoluble fraction; the corresponding figures for the ATPase reaction were 1% and 78%.

Effects of certain agents. These are given in Table 6. Ouabain at 0.13mm concentration failed to overcome the inhibition of the bindings shown by the Na⁺ ions. Inhibition of binding given by *p*-chloromercuribenzoate (0.01mm) was overcome by cysteine and cysteine itself slightly enhanced the reaction; it would seem therefore that SH groups are to some extent involved in the phosphate binding. A fairly high concentration (0.2mm) of chlorpromazine, on the other hand, facilitated the binding reaction while markedly inhibiting the ATPase; here competition for substrate may be the principal factor.

DISCUSSION

Interpretation of the nature of the structures accepting phosphate from ATP in these experiments is limited by our rudimentary knowledge of the

tissue phosphoproteins. A considerable part of the incorporation probably occurs into non-enzymic phosphoproteins of the classical type, represented by casein and phosvitin, through the mediation of the Mg²⁺-dependent protein kinase enzyme (or enzymes) that occur in cerebral microsomes (Rodnight & Lavin, 1964). This enzyme can phosphorylate casein and phosvitin, but is without action on phosphoproteins that function as enzymes (Rabinowitz & Lipmann, 1960). Phosphoproteins of the classical type have yet to be isolated from mammalian tissues, but there is suggestive evidence for their occurrence in brain (Heald, 1961; Rodnight, 1966). Moreover the presence in a cerebral membrane fraction of a natural acceptor for the protein kinase enzyme has been shown by Kothary (1964): with Mg-[³²P]ATP as precursor, phosphorylserine residues in a protein fraction isolated from ox-brain particulate matter were labelled only in the presence of a partially purified protein kinase enzyme prepared from the same source. A similar result has been reported for a different protein fraction by Matsui, Orikabe, Ishikawa & Shimazono (1965). It may be noted, however, that when acting on phosvitin the protein kinase enzyme is not inhibited by thiol reagents (Rabinowitz & Lipmann, 1960), whereas in the present study incorporation of phosphate into intrinsic structures was to some extent reduced by *p*-chloromercuribenzoate; this would suggest that other systems are also involved in phosphate binding under these conditions. These may include phosphorylated enzymes (particularly of the type represented by glycogen phosphorylase in its active form) and their respective kinases. Phosphotransferases such as adenylate kinase and nucleoside diphosphate kinase and phosphatase also

occur in microsomes and may involve a phosphorylated serine group in their mechanism of action.

With regard to the inhibition of the binding given by univalent cations, Matsui *et al.* (1965) observed that Na^+ and K^+ inhibited phosphate binding from ATP in the insoluble residue remaining after extraction of an acetone-dried powder of whole brain with buffers. This may be the same effect as reported in the present work, but the different nature of the preparation makes a comparison uncertain.

With regard to our earlier observations on the labelling of protein-bound phosphorylserine groups in slices of cerebral tissue (see the introduction for details), the present findings do no more than demonstrate that ATP can function as a donor for the phosphorylation of such membrane-located serine residues; there is every reason to believe therefore that this is also the situation in the intact tissue. Further understanding of the problem will involve isolating and comparing the structures of the acceptor molecules that react with ATP before and after disruption of the cell.

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