Properties of Phosphate Bound to Cerebral Microsomes during Adenosine-Triphosphatase Activity

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1. Ox-brain microsomes were incubated with [γ - ^{32}P]ATP under various conditions. After the reaction, which was stopped with trichloroacetic acid, a small amount of phosphate remained bound to the washed precipitate. 2. Properties of the bound phosphate were studied by treatment with buffers and solvents. 3. The Na^+ -dependent increment in bound phosphate, predominant at low ATP concentration and features of which suggest involvement in the concomitant adenosine-triphosphatase activity, was rapidly released in both circumstances. 4. In aqueous media the labile phosphate was released entirely as inorganic phosphate at faster rates with increasing alkalinity. 5. In acidified chloroform-alcohol mixtures the released phosphate appeared both as inorganic phosphate and different single ^{32}P -labelled organic phosphates, which were tentatively identified as the relevant mono-alkyl phosphates, presumably derived by acid-catalysed alcoholysis of a labelled microsomal component, or components. 6. The labile phosphate corresponded to the P exchangeable with non-radioactive ATP added during the enzyme reaction. 7. The possible molecular nature of the labile fraction of the bound phosphate is discussed.

In the preceding paper Rodnight, Hems & Lavin (1966) showed that when microsomes from guinea-pig or ox-brain cortex are incubated with [γ - ^{32}P]ATP and then denatured with acid, the precipitate is found to contain a small amount of bound phosphate, stable to repeated washing at 0° with further acid and with solvents. There appeared to be two types of bound phosphate: an unstable fraction dependent on Na^+ for its formation, possessing several features suggesting it is intimately related to the $(\text{Na}^+, \text{K}^+)\text{-ATPase}^*$ activity of the preparation, and a relatively stable fraction requiring only Mg^{2+} for its appearance. The occurrence of Na^+ -dependent bound phosphate has been described in other membrane preparations from brain (Nagano *et al.* 1965; Gibbs, Roddy & Titus, 1965; Hokin, Sastry, Galsworthy & Yoda, 1965; Skou, 1965) and other tissues (Post, Sen & Rosenthal, 1965; Albers, Fahn & Koval, 1963).

The present paper describes experiments which demonstrate that the Na^+ -dependent fraction of the bound phosphate is covalently linked to a component (or components) of cerebral microsomes to form a 'high-energy' phosphate. A preliminary

account of the work has already appeared (Hems & Rodnight, 1965).

EXPERIMENTAL

Preparation of microsomal fractions. Ox-brain cerebral grey matter microsomes were prepared by differential centrifugation in 0.32M -sucrose containing 0.5mM -EDTA as described by Rodnight *et al.* (1966).

Materials. Reagent-grade materials were used for preparation of all buffers, media and solvents. Monomethyl phosphate (sodium salt) was obtained from Koch-Light Ltd., Colnbrook, Bucks. ATP (disodium salt) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. and converted where required into the tris salt by using Dowex 50 (H^+ form).

Radioactive chemicals. [^{32}P]P_i in dilute HCl was obtained from The Radiochemical Centre, Amersham, Bucks. [γ - ^{32}P]ATP (tris salt) was prepared as described by Rodnight & Lavin (1966); specific radioactivity was $0.1\text{--}0.4\text{mCi}/\mu\text{mole}$. Ethyl or methyl monoalkyl [^{32}P]phosphates were prepared from [^{32}P]ATP employing the alcoholic system of Eichberg & Dawson (1964) as follows: a small volume of high specific radioactivity [^{32}P]ATP was added to more than 100 vol. of chloroform-alcohol (2:1, v/v) acidified with conc. HCl (see below for concentrations) at 37° for about 3 hr. The solvent was then washed twice with water; the aqueous extract contained [^{32}P]P_i and [^{32}P]ATP, and also mono-alkyl [^{32}P]phosphate. The yield of the latter was about 30% in chloroform-methanol containing conc. HCl

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

(0.33%, v/v), and about 15% in chloroform-ethanol acidified with conc. HCl (1%, v/v) (Table 2); the higher acid concentration in the latter system increased the yield of ethyl phosphate. Where required, labelled methyl phosphate was eluted from paper chromatograms after separation from ATP and P_i in the phenol solvent described below.

Analytical methods. Microsomal protein was determined by a biuret method, with crystalline bovine plasma albumin used as standard. P_i was extracted as a complex with molybdate into isobutanol-benzene (1:1, v/v) as described by Rodnight *et al.* (1966). In procedures where [^{32}P] P_i was extracted by this method, quantitative recovery of carrier P_i was demonstrated by reduction of the complex with $SnCl_2$ and reading at 730 m μ on a Unicam SP.600 spectrophotometer.

Paper chromatography of extracts of labelled microsomal precipitate and synthetic phosphates was carried out in the following systems. (a) Descending, on Whatman no. 1 paper, with phenol saturated with HCl-acetic acid-ethanol (50:5:6, by vol.). (b) Ascending, on Whatman no. 20 paper, in ethanol-12% (w/v) NH_3 (3:2, v/v). Both of these solvents were used by Eichberg & Dawson (1964). (c) Ascending, on Whatman no. 20 paper, in propan-2-ol-aq. NH_3 (sp.gr. 0.88)-water (6:1:3, by vol.), as used by Bader, Post & Sen (1965). Column chromatography was on Dowex 1 (X8; 200-400 mesh; formate form), eluting with 0.02 M-ammonium formate in 4 N-formic acid. Electrophoresis was on Whatman no. 1 paper with acetic acid-formic acid buffer, pH 1.8, for 4 hr. at 8 v/cm. Paper strips were stained for phosphate by the method of Wade & Morgan (1953).

Incubation of microsomes with [^{32}P]ATP. This was carried out in 2.5 ml. at 37° with 1 mg. of microsomal protein, in the presence or absence of Na^+ , usually 15 mM. [^{32}P]ATP concentration was usually 20 μ M. Other reaction conditions, mixing, terminating with trichloroacetic acid, washing of precipitates with acid, and estimation of ATP hydrolysis, were as described by Rodnight *et al.* (1966).

Treatment of acid-washed labelled precipitates with buffers and solvents. The following procedures were used. (a) Buffers: after three washes in 10% (w/v) trichloroacetic acid containing $m\text{-}H_3PO_4$, a fourth wash was carried out in 10% (w/v) trichloroacetic acid with no added H_3PO_4 . The precipitate was then suspended in 2 ml. of buffer, usually at 22° and agitated for a given period, at the end of which 100% (w/v) trichloroacetic acid was added to final pH less than 2. The suspension was cooled to 0°, spun for 20000 g-min. and the supernatant extracted for P_i , a suitable amount of carrier having been carried over from the deposit from the third wash. Tris-HCl buffers (0.1 M) were used at pH 7.3 and 8.3 and 0.1 M-succinic acid brought to pH 6.0 with NaOH produced a final pH 5.6 on resuspension of the pellet. Neutralized hydroxylamine-HCl was made fresh as described in Table 1. (b) Solvents. After three acid washes ethanol-ether (1:1, v/v) was used for the fourth wash of the labelled precipitate (1.5 ml. at 0°), which was then suspended in 3 ml. of chloroform-alcohol acidified with conc. HCl. Acid concentration was 0.33% (v/v) in chloroform-methanol and 1% (v/v) in chloroform-ethanol, as a result of the observation on the yield of labelled alkyl phosphate in the system described above. The suspension, at 37°, was manually shaken at intervals; 2 hr. brought about complete release of labile P_i . It was then cooled to 0°, centrifuged for 20000 g-

min. and decanted carefully. The supernatant was shaken for 15 sec. with 4 ml. of water and 4 ml. of upper phase (HCl-alcohol-water) taken for P_i extraction as described above. Less than 10% of the original supernatant counts remained in the organic phase. Bound P_i was thus fractionated into that which remained in the precipitate and that released as P_i or organic P_i . If the extract was to be used for chromatography or electrophoresis, the organic phase was washed once with water, the aqueous extracts combined and freeze-dried, and then taken up in a small volume of water.

Measurement of radioactivity. Liquid samples were counted in a 10 ml. M6H Geiger-Müller counter (20th Century Electronics Ltd., New Addington, Surrey). Paper strips were scanned for ^{32}P with an end-window Geiger-Müller tube (20th Century Electronics Ltd.); successive 0.5 cm. sections were counted through an aperture in a 3 mm.-thick Perspex plate (3 cm. $\times$ 0.5 cm.), without cutting up the strip.

RESULTS

Lability of bound phosphate in aqueous media. Phosphate transferred to microsomes during the reaction with 20 μ M-ATP and Na^+ described by Rodnight *et al.* (1966) was found to be unstable in aqueous media. Thus when the denatured and acid-washed microsomes were suspended in aqueous buffers the major part of the bound P_i was fairly rapidly released as [^{32}P] P_i by a pH- and temperature-dependent reaction (Fig. 1). About 70% of

Fig. 1. Release of bound P_i in aqueous media. Microsomes were incubated with [^{32}P]ATP (20 μ M) and 15 mM- $NaCl$ as described in the text; the reaction was in 2.5 ml. at 37° for 10 sec. and was stopped with trichloroacetic acid. Precipitates were washed four times with trichloroacetic acid and then agitated at 22° in different buffers. The treatment with buffers was terminated by cooling to 0° and adding trichloroacetic acid to pH less than 2; the suspension was then centrifuged and the ^{32}P -labelled substances were fractionated as described in the text. ●—●, Release by treatment at pH 8.3; ○ - - - ○, pH 8.3 at 0°; ■, pH 7.3; ▼, pH 5.6; ▲, 5% trichloroacetic acid (pH 0.7).

the total P bound during 10 sec. reaction was released in 90 min. at pH 8.3 and 22°. The remainder was relatively stable; about one half was released if incubation was prolonged for 18 hr. The rate of release decreased as the pH was lowered over the range 8.3–0.7. Similar results have been reported for other preparations of microsomes from brain by Nagano *et al.* (1965) and Hokin *et al.* (1965), and from kidney by Bader, Sen & Post (1966). In view of its lability it is worth emphasizing that the bound P in our experiments was sufficiently stable at 0° and pH 0.5 (10% trichloroacetic acid in *m*-phosphoric acid), to permit centrifugal washing, as established by Rodnight *et al.* (1966).

The release of bound P was not dependent on the ionic strength of the buffer, or on the concentration of P_i present, and it was not increased if the suspension was agitated. Suspension of the acid-washed precipitate in neutral chloroform-methanol (2:1, v/v) at 0° for 10 min. to extract lipids [a procedure shown by Rodnight *et al.* (1966) to remove less than 15% of the total bound P] did not significantly affect the subsequent rate of release of bound P in buffers.

As has been noted by other workers (Nagano *et al.* 1965; Hokin *et al.* 1965; Bader *et al.* 1966) in different microsomal systems, the lability of the Na^+ -dependent bound P in aqueous media was greatly increased in the presence of hydroxylamine (Table 1).

Lability of bound phosphate in acidified chloroform-alcohol mixtures. In contrast with its relative stability in neutral chloroform-methanol (2:1, v/v), the Na^+ -dependent bound P was unstable if the same solvent was acidified, the rate of release at 37° being comparable with that in aqueous media (Figs. 1 and 2). However, in this case the P did not all appear as P_i ; about 50–60% was so released, but the remainder of the labile P appeared as another ^{32}P -labelled substance yielding a separate single peak on paper and column chromatography. A similar observation has been made with kidney microsomal material (Bader *et al.* 1966). From

evidence presented below, this ester appears to be monomethyl [^{32}P]phosphate, derived by methanolysis of a component, or components, in the labelled microsomal precipitate.

The release of Na^+ -dependent bound P in acidified chloroform-methanol showed several features similar to those seen with aqueous media (Fig. 2). As in the latter case, about 70% was released relatively rapidly, following a similar time-course, and showing temperature-dependence. Chloroform was not essential for the release of the phosphate, which occurred as rapidly in methanol-conc. hydrochloric acid (200:1, v/v).

Rodnight *et al.* (1966) showed that the Na^+ -dependent level of P binding with 20 μM -ATP is maximal within 1 sec. of starting the reaction and declines after some 50% of the ATP has been split to a level approaching that of the zero-time controls; in the absence of added Na^+ , on the other hand, the maximum level of binding is reached later and is more stable. It was therefore of interest to compare the lability of the P bound under these two conditions. The acid-denatured precipitates, labelled for various reaction times with 20 μM -[^{32}P]ATP, were suspended in acidified chloroform-methanol for 2 hr. at 37° and the total released P was determined. It was found that the Na^+ -dependent increment in

Fig. 2. Release of bound P in chloroform-methanol mixtures. Conditions of P binding were as in Fig. 1. The precipitates were washed three times with trichloroacetic acid, once with 1.5 ml. of ethanol-ether (1:1, v/v) at 0°, and then suspended in 3 ml. of solvent, at 37° unless indicated, with occasional manual agitation. At the end of the solvent treatment the suspension was cooled to 0°, centrifuged and ^{32}P -labelled substances were extracted for fractionation as described in the text. ▲, Total P released by treatment with chloroform-methanol (2:1, v/v); ■, chloroform-methanol-conc. HCl (200:100:1, by vol.), 0°; ●—●, chloroform-methanol-conc. HCl; ○—○, release of P_i only by chloroform-methanol-conc. HCl.

Table 1. *Effect of hydroxylamine on release of bound phosphate by aqueous media*

Microsomes were incubated with [^{32}P]ATP as in Fig. 1. The washed precipitates were suspended in 5% (w/v) hydroxylamine-HCl (adjusted to pH 5.9 with tris) or in 5% (w/v) NH_4Cl in 0.5 *M* tris-HCl (final pH 5.9) at 0° for 20 min. The bound P was fractionated as described in the text.

Treatment	Bound P (μ moles)		
	Total	Remaining in precipitate	Released as P_i
Hydroxylamine-HCl	28	4.9	21.6
NH_4Cl	24.9	22.9	1.5

the bound P was all released, whereas only 30–50% of the P bound in the absence of Na⁺ was labile under these conditions (Fig. 3a). An identical result was obtained with 0.1M-tris hydrochloric

acid, pH 7.3, as the releasing agent and it was evident that whichever method was employed to release the bound P, the labile fraction was similar in amount, and corresponded approximately to the Na⁺-dependent increment in the binding.

If chloroform-ethanol-conc. hydrochloric acid (200:100:3, by vol.) was used instead of acidified chloroform-methanol to extract the labelled precipitates, the major portion of the Na⁺-dependent bound P was again released into the solvent as P_i and a ³²P-labelled ester (Table 2); however, this ester was different from the one found in the methanol solvent and its yield was less (about 15% of the total bound P) than in the former case. Chromatographic evidence, presented below, suggested that the ethanol-extracted substance was monoethyl [³²P]phosphate, again presumably derived by alcoholysis.

The lability in acidified chloroform-methanol of P bound as a result of incubating microsomes in 20μM-ATP under various conditions at 37° for 10 sec. is shown in Table 3. This reproduces several of the findings, both with respect to factors influencing total bound P and the ATPase reaction, described by Rodnight *et al.* (1966). The reaction in the presence of ouabain was carried out without preincubation with the glycoside. The major part of the P bound under each condition was labile in acidified chloroform-methanol. Although it is not shown in Table 3, a similar fraction of the bound P in each case was released by treatment with 0.1M-tris-hydrochloric acid, pH 7.3, at 37° for 2 hr. This correspondence in the amount of bound P labile in either buffer or acidified chloroform-alcohol mixtures was in fact found whenever both methods were used to release P from precipitate, regardless of the conditions of labelling.

Incubation of microsomes with concentrations of ATP higher than 20μM enhances the rate and

Fig. 3. Release by acidified chloroform-methanol of P bound after various reaction times. Microsomes were incubated with [³²P]ATP as described in the text. The acid-washed precipitates were treated with chloroform-methanol-conc. HCl (200:100:1, by vol.) at 37° for 2 hr. as described in Fig. 2. Filled-in symbols represent original total bound P, open symbols bound P remaining after treatment, derived from the difference between original total bound P and released P. ●, ○, In the presence of NaCl (15 mM); ■, □, without NaCl. (a) Reaction with 20 μM-ATP. (b) Reaction with 200 μM-ATP (symbols as in a).

Table 2. Treatment of bound phosphate and ATP with acidified chloroform-alcohol mixtures

Incubation of microsomes with 20 μM-[³²P]ATP for 10 sec. at 37°, and preparation of monoalkyl [³²P]phosphates, were as described in the text. The washed precipitates were treated with solvent for 2 hr. as described in Fig. 2. Bound P was fractionated as described in the text; yield of alkyl P was calculated from chromatographic evidence.

Solvent	NaCl (mM)	Bound P				ATP
		Total (μmoles)	Remaining in precipitate (%)	Released as P _i (%)	Released as ³² P-labelled ester (%)	
Chloroform-methanol-conc. HCl (200:100:1, by vol.)	15	35	26	37	37	30
As above	100	43	16	49	35	
Chloroform-ethanol-conc. HCl (200:100:3, by vol.)	15	36	33	47	20	15
As above	100	50	16	66	18	

Table 3. Release by acidified chloroform-methanol of phosphate bound under various conditions

Microsomes (1 mg. of protein) were incubated with [32 P]ATP (20 μ M) in 2.5 ml. at 37° for 10 sec. unless indicated. The washed precipitates were treated with chloroform-methanol-conc. HCl (200:100:1, by vol.) for 2 hr. at 37° as described in Fig. 2. Bound P was fractionated as described in the text.

Univalent cations (mM)	Other conditions	Bound P (μ moles)			ATP hydrolysed (%)
		Remaining in precipitate	Released as P_i	Released as 32 P-labelled ester	
—	—	7	3	5	13
Na ⁺ , 15	—	7	12	11	35
Na ⁺ , 100	—	9	18	20	13
Na ⁺ , 15; K ⁺ , 2	—	8	5	8	41
Na ⁺ , 100; K ⁺ , 15	—	5	9	7	36
Na ⁺ , 15	Ouabain, 10 ⁻⁴ M	10	13	17	17
Na ⁺ , 15	0° for 1 min.	7	9	9	7

amount of P binding occurring with Mg²⁺ alone, but progressively reduces the Na⁺-dependent increment in the total binding and the proportion of the latter that turns over rapidly (Rodnight *et al.* 1966). It was therefore decided to examine the lability of bound P at higher ATP concentrations. At a concentration of 200 μ M less than 50% of the total labelling found in the presence of Na⁺ after incubation for 10 sec. was labile in acidified chloroform-methanol; however, as before, the whole of the Na⁺-dependent increment appeared to be of this type (Fig. 3b). The Figure also shows that only about 20% of the labelling in the absence of Na⁺ was labile at the same time-interval. Although the relative amount of labile P was substantially less when 200 μ M-ATP was used, in absolute terms it was approximately twice as great as that found at the lower substrate concentration.

Tentative identification of 32 P-labelled esters. The labelled substances produced by treatment of the microsomal precipitate with acidified methanol or ethanol mixtures could be separated from P_i by paper chromatography, in each case as a single peak. These peaks were indistinguishable respectively from those given by methyl and ethyl monoalkyl phosphates; chromatography of extracts of precipitates labelled as a result of incubation of microsomes in 20 μ M-ATP is illustrated in Fig. 4. Of the two 32 P-labelled substances, that released in acidified chloroform-methanol was the more extensively investigated. Chromatography of the extract of precipitate labelled as in Fig. 4 with 20 μ M-ATP in three solvent systems on paper, or on Dowex 1 (X8; formate form), with monomethyl phosphate (sodium or tris salt) or methyl [32 P]-phosphate (tris salt) used as reference substances, revealed identical behaviour in all cases. A similar result was obtained by electrophoresis on paper at pH 1.8. The phenol solvent paper system produced the best separation of the two peaks given by the

Fig. 4. Paper chromatography of acidified chloroform-alcohol extracts of labelled microsomal precipitate. Conditions of P binding were as in Fig. 1, except that the reaction was carried out on a larger scale (3–5 mg. of microsomal protein) to provide sufficient P for chromatography. The acid-washed precipitate was suspended in solvent for 2 hr. at 37° and the extracts were prepared for chromatography in the phenol solvent as described in the text. Continuous lines represent microsomal extracts; broken lines monoalkyl [32 P]phosphates (counts/min. $\times 0.1$). ●, Chloroform-methanol-conc. HCl (200:100:1, by vol.); ○, chloroform-ethanol-conc. HCl (200:100:3, by vol.). The slow-moving peak of P_i from the ethanol extract, and alternate experimental points in all cases, are omitted for clarity.

extracts and of the respective monoalkyl phosphates and labelled substances from each other (Fig. 4). R_f values and peak elution volumes found in the chromatographic system are given in Table 4.

Having postulated that the 32 P-labelled substances obtained by solvent treatment were monoalkyl phosphates, we considered further properties of these compounds with a view to more definitive identification. One characteristic is the

Table 4. *Chromatography of acidified chloroform–alcohol extracts of labelled microsomal precipitates*

The extracts were obtained as described in Fig. 2. Full details of the chromatographic procedures and solvents are given in the text. The alcohol used to derive the extract containing a given labelled substance is indicated in the Table.

Paper chromatography (R_F values)

Solvent	P_i	ATP	Methyl [^{32}P]phosphate	Methyl phosphate (Na salt)	^{32}P -labelled substance (from methanol)	Ethyl [^{32}P]phosphate	^{32}P -labelled substance (from ethanol)
Ethanol-NH ₃	—	0.22	0.45	0.44	0.45	—	—
Propan-2-ol-NH ₃	—	0.30	0.49	0.47	0.49	—	—
Phenol	0.08	0.06	0.54	0.49	0.54	0.66	0.65

Column chromatography
(peak elution volume, ml.)

Resin			
Dowex 1 (formate form)	155	205	205

pH-dependence of their stability to hydrolysis, which has been described for monomethyl phosphate by Bunton, Llewellyn, Oldham & Vernon (1958). This property of the substance released by acidified chloroform–methanol was therefore compared with that of monomethyl [^{32}P]phosphate; the latter was prepared as described in the Experimental section, and [^{32}P]ATP removed from the solution by passage through a column of deactivated charcoal. Both labelled phosphates were then separated from [^{32}P]P_i by barium fractionation; two precipitations were carried out with carrier potassium dihydrogen phosphate present and then excess of Ba²⁺ was precipitated with sodium sulphate. Hydrolysis was carried out in stoppered tubes at 100° in five different media: N- and 0.2N-perchloric acid, and buffers at pH 4.3, 6.1 and 7.7 (measured at 20°) containing, in addition to sodium hydroxide, 0.05M-acetate, succinate and borate, and in the last case 0.1M-potassium chloride (see Bunton *et al.* 1958). During the hydrolysis, portions were withdrawn at intervals, cooled, 1 μmole of potassium dihydrogen phosphate was added as carrier, and P_i extracted as phosphomolybdate into isobutanol–benzene (1:1, v/v); both phases were then counted, and the percentage hydrolysis was calculated. It was found that in all cases the hydrolysis was compatible with the first-order criterion, and rate constants were derived graphically. These did not differ significantly from those of synthetic monomethyl phosphate (Fig. 5, the reference curve having been taken from Bunton *et al.* 1958, without correction for the effect of temperature on pH).

The above characterization by chromatographic and hydrolytic methods was concerned only with the single ^{32}P -labelled substances produced by treatment of precipitates with acidified chloroform–alcohol mixtures after labelling at 37° with

Fig. 5. Dependence on pH of the rate of hydrolysis of the ^{32}P -labelled ester derived by acidified chloroform–methanol extraction of the labelled microsomal precipitate. The unknown substance was obtained as in Fig. 4 and purified, as was monomethyl [^{32}P]phosphate, as described in the text. Details of the hydrolysis and derivation of first-order rate constants are given in the text. The abscissa indicates pH at 20°, although hydrolysis was carried out at 100°. The broken line is the pH–hydrolysis profile of synthetic methyl phosphate, taken from Bunton *et al.* (1958), without modification. ●, ^{32}P -labelled substance; ○, monomethyl [^{32}P]phosphate.

20 μM-[^{32}P]ATP in the presence of Na⁺. In the previous section, measurement of lability of bound P produced under various conditions was described and it was noted that the labile bound P was always released as approximately equal amounts of P_i and organic P (Table 3). Chromatographic characterization of the labile organic P derived under these different conditions was carried out in the phenol system. In all cases, whatever the proportion of

bound P released, the organic P appeared as a single peak corresponding to that given by monomethyl phosphate; this was shown for labelling in $20\text{ }\mu\text{M}$ - or $200\text{ }\mu\text{M}$ -ATP, with or without added Na^+ and in the presence of 0.1 mM -ouabain and Na^+ at the lower substrate concentration.

DISCUSSION

The very small amounts of unknown ^{32}P -labelled esters produced by treatment of the labelled microsomal precipitate with acidified chloroform-alcohol mixtures were insufficient for final identification but the chromatographic and hydrolytic evidence strongly supports the assumption that they are monoalkyl phosphates. These substances are presumably formed by alcoholysis of a phosphorylated component in the microsomes (measured in our experiments by the labile fraction of the bound P) rather than by phosphorylation of an intrinsic labile alkyl group during the enzyme reaction. The latter and other alternative explanations for the findings require the postulation of at least two types of labile bound P possessing several most unlikely sets of coincidental properties. The present results therefore confirm with additional data our earlier conclusion (Hems & Rodnight, 1965) regarding the origin of monomethyl phosphate in the system. The same conclusion has been reached by Bader *et al.* (1966) with respect to their observations on a labelled preparation of kidney membrane fragments.

Acid-catalysed alcoholysis of ATP has been described by Eichberg & Dawson (1964): transfer of phosphate to methanol or ethanol required only hydrochloric acid and in this and quantitative respects resembled the present findings (see Table 2). The possibility that the labile bound P is derived from bound ATP has been excluded by measurement of ATP binding (Rodnight *et al.* 1966) and is further discounted in the present work by the lability of the bound P in aqueous solutions. Since Eichberg & Dawson (1964) were unable to demonstrate an acid-catalysed methanolysis of ADP, AMP, creatine phosphate, pyrophosphate or P_i , and because of the similarities in yield of alkyl phosphates in the two experimental situations, our findings suggest that the phosphate is bound in a covalent linkage of 'high-energy' type. 'High-energy' properties have been suggested for the similar bound P fraction of kidney microsomes (Bader *et al.* 1966).

A possible explanation for a loss of bound P as organic phosphate in acidified chloroform-alcohol mixtures would be extraction of labelled 'phosphatidopeptides' (Folch & LeBaron, 1959). The phosphorylated components of this fraction are polyphosphoinositides and possibly peptides;

the former are well known to have a high phosphate turnover (Hawthorne & Kemp, 1964). However, they are excluded from consideration as the labile organic P in the present work, not only by its chromatographic and hydrolytic behaviour but by the properties of the component in aqueous media and the fact that acidified alcohol alone releases the labile P.

Similar observations to ours on the lability of the Na^+ -dependent bound P in aqueous solutions have been reported for several different membrane preparations by Albers *et al.* (1963), Nagano *et al.* (1965), Hokin *et al.* (1965) and Bader *et al.* (1966). On the basis of its increased lability in hydroxylamine and from studies of its pH profile of hydrolysis and sensitivity to an acyl phosphatase, the latter three groups have suggested that the phosphate is esterified to an acyl acceptor. The present data are also consistent with this hypothesis, for the free energy of hydrolysis of acyl phosphates is generally high. It seems very probable that the acyl phosphate is bound to protein since labelled peptides have been demonstrated in peptic digests of membrane fragments after incubation with ^{32}P -ATP (Albers *et al.* 1963; Hokin *et al.* 1965; Bader *et al.* 1966). The phosphate acceptor might then either be a C-terminal amino acid or a side-chain carboxyl group of glutamic acid or aspartic acid residues. It is noteworthy that Imamura, Kanazawa, Tada & Tonomura (1965) have reported evidence for the transitory phosphorylation of glutamic acid or aspartic acid carboxyl groups during the splitting of ATP by actomyosin.

In the preceding paper we demonstrated turnover of part of the total bound P (or its precursor) by addition of non-radioactive ATP to the reaction mixture during the course of ATP hydrolysis (Rodnight *et al.* 1966). It was concluded that the unstable component most probably represents a phosphorylated intermediate in ATPase activity. This exchangeable fraction of the bound P corresponds in amount to the labile component described in the present paper; this can be seen to be the case at two different ATP concentrations, and during all stages of the reactions in the presence or absence of Na^+ . This fact and the apparent 'high-energy' nature of the component corroborate the above conclusion, which, if considered with the widely postulated involvement of ATP hydrolysis in active movement of cations (McIlwain, 1963; Skou, 1965), seems to lend support to transport mechanisms involving phosphorylated components of the membrane rather than those not implicating such intermediates.

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