Separation at Neutral pH Value of ^{32}P -Labelled Proteins in a Membrane Preparation from Ox Brain

PARTIAL CHARACTERIZATION OF A COMPONENT OF THE SODIUM-PLUS-POTASSIUM ION-ACTIVATED ADENOSINE TRIPHOSPHATASE

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1. Ox brain microsomal fractions were labelled with [^{32}P]ATP in the presence of Na^+ and the reaction was stopped with sodium dodecyl sulphate. The Na^+ -dependent bound phosphate was isolated on Sephadex G-25 and by acetone precipitation. The bound phosphate isolated under these neutral conditions was labile to hydroxylamine and gave the same pH profile of hydrolysis as that isolated by precipitation with strong acids. 2. When membrane protein was labelled with [^{32}P]ATP, solubilized with sodium dodecyl sulphate and fractionated on Sepharose 6B, the Na^+ -dependent label emerged in a peak corresponding to protein of molecular weight 570000–580000. On fractionation of this protein peak on polyacrylamide gels containing detergent and urea, the Na^+ -dependent label occurred in a single band corresponding to a protein of molecular weight 102000. 3. Fractionation on Sepharose 6B of protein labelled with [^{32}P]ATP in the absence of Na^+ revealed three labelled peaks, one of which corresponded in position to the Na^+ -dependent label. Electrophoresis of this peak material on polyacrylamide gels showed that most of the label occurred in two fast-running bands. Cyclic AMP stimulated the labelling in these two bands, but had no effect on the labelling of the band corresponding in position to the Na^+ -dependent label. 4. Di-isopropyl [^{32}P]phosphorofluoridate also labelled the band corresponding to the Na^+ -dependent label on gel electrophoresis. The labelling of this band by the reagent was inhibited by 50–60% by 3 mM-ATP, but there was no evidence to suggest that the group labelled is normally phosphorylated by ATP.

The hydrolysis of ATP by the membrane-bound Na^+, K^+ -ATPase† system is known to involve the transfer by a Na^+ -dependent reaction of the γ -phosphoryl group of ATP to a protein component of the membrane (for review see Bonting, 1970). The present study had the object of exploring certain aspects of this reaction in a membrane preparation made from cerebral cortex of the ox.

First, we wished to confirm the acyl nature of the bond formed by the Na^+ -dependent reaction at neutral pH. In acid-denatured membrane protein the phosphate was shown to be covalently bound to the γ -carboxyl group of a glutamic acid residue in an acyl linkage (Kahlenberg *et al.*, 1969); the bond was also shown to possess the properties of an acyl phosphate after denaturation of the enzyme with sodium dodecyl sulphate followed by precipitation with acid (Nagano *et al.*, 1967). The use of acid in these studies to denature or precipitate the labelled protein, however,

limits the conclusions that can be drawn about the nature of the bond formed by the enzyme at neutral pH, since the possibility of an acid-catalysed migration of phosphate cannot be excluded. Shifts of dialkylphosphoryl groups from N to O at acid pH were observed in *N*-di-isopropylphosphoryl derivatives of serine and threonine (Plapinger & Wagner-Jauregg, 1953). It seemed necessary therefore to attempt to characterize the bond in labelled protein that had not been at any stage exposed to extremes of pH. In the present work this was achieved by terminating the reaction with sodium dodecyl sulphate, which as Nagano *et al.* (1967) and Rodnight (1970) showed solubilizes the membrane protein and stabilizes the Na^+ -dependent labelling, and then isolating the labelled protein by gel filtration and precipitation with acetone.

Secondly, we wished to fractionate the detergent-treated labelled membrane protein by gel filtration and electrophoresis with the object of purifying the component that accepts phosphate in the Na^+ -dependent reaction and obtaining an estimate of its molecular weight. The values determined for the

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† Abbreviation: Na^+, K^+ -ATPase, sodium-plus-potassium ion-activated adenosine triphosphatase.

latter parameter agreed well with those reported by other workers for different membrane preparations.

Finally we decided to explore the possibility that at some stage in the hydrolysis of ATP by the enzyme a serine hydroxyl group is phosphorylated by ATP through a reaction stimulated by cyclic AMP. The membrane preparation used in the present work contains protein kinase activity towards endogenous acceptors that is stimulated by cyclic AMP (Weller & Rodnight, 1973). The physiological function of this system is poorly understood (Rodnight, 1971) and the possibility that it is part of a control mechanism for the Na^+, K^+ -ATPase has not been excluded. The involvement of a serine residue at the active site of the latter enzyme is further suggested by the ability of di-isopropyl phosphorofluoridate and similar reagents to inhibit the enzyme (Hokin & Yoda, 1964; Sachs *et al.*, 1969), although in this case there is no evidence that the residue is phosphorylated by ATP. The present study has shown that the component of the enzyme system that accepts phosphate in the Na^+ -dependent reaction is not phosphorylated by a cyclic AMP-stimulated protein kinase reaction, although it does appear to be phosphorylated by di-isopropyl phosphorofluoridate.

A preliminary account of this work has been given (Alexander & Rodnight, 1970).

Experimental

Materials

Sodium dodecyl sulphate was obtained from Sigma (London) Chemical Co., London S.W.6, U.K., and sodium decyl sulphate was from Mann Research Laboratories, New York, N.Y., U.S.A.; urea was from British Drug Houses Ltd., Poole, Dorset, U.K.; hydroxylamine hydrochloride was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K., and was neutralized to pH 7.0 with Tris before use and if not used at once was stored at 2°C for a maximum of 24 h.

Acrylamide and *NN'*-methylenebisacrylamide were purchased from Kodak Ltd., Kirby, Liverpool, U.K. Blue Dextran 2000, Sephadex G-25 and Sepharose 6B were supplied by Pharmacia (G.B.) Ltd., London W.5, U.K.

[^{32}P]ATP labelled in the γ -position was prepared as described by Rodnight & Lavin (1966). Di-isopropyl [^{32}P]phosphorofluoridate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Membrane preparation

A partially purified microsomal fraction from ox brain was prepared by the method of Rodnight (1970). The preparation was stored at -20°C in 4 mM-imidazole-HCl buffer (pH 7.2) at a concentration of about 10 mg of protein/ml. In some experiments the

preparation was centrifugally washed to decrease its content of bound cations by the procedure of Goldfarb & Rodnight (1970).

Methods

Protein was determined by the method of Miller (1959), with bovine plasma albumin as standard.

Labelling of microsomal fractions with [^{32}P]ATP. Two procedures were used, *A* and *B*; the general conditions, mode of stirring etc. were those of Rodnight (1970). In procedure *A* the reaction mixture contained 30 mM-Tris-HCl (pH 7.4), 2.5 μM - MgCl_2 , 2.5 μM -[^{32}P]ATP (10.5×10^3 – 12.5×10^3 c.p.m.), 50 mM-NaCl and 0.5 mg of microsomal protein in a final volume of 2.5 ml. The reaction was started by addition to the rapidly stirred medium of 0.25 ml of protein suspension and stopped by addition of 0.25 ml of 1% (w/v) sodium dodecyl sulphate containing 0.1% Blue Dextran, giving a final detergent concentration of 0.092% and a detergent/protein ratio (in mg) of 5:1. Procedure *B* was used when a higher yield of labelled protein for fractionation was required. The reaction mixture contained, in the same volume, 30 mM-Tris-HCl (pH 7.4), 50 μM - MgCl_2 , 50 μM -[^{32}P]ATP, 50 mM-NaCl and 5–10 mg of protein. The reaction was started by the rapid addition (from a syringe) of 0.6–1.0 ml of protein suspension and stopped within 1 s with 0.25 ml of 50% (w/v) sodium dodecyl sulphate, giving a detergent/protein ratio (in mg) of 18:1.

Gel filtration. Columns supplied by Pharmacia (G.B.) Ltd. with an internal diameter of 2.5 cm were used. The length of the gel in the Sephadex G-25 columns was 36 cm and that in the Sepharose 6B columns was 38 cm. The columns were operated by upward displacement by using a multichannel peristaltic pump to apply sample and eluent. The absorbance of the column effluent was monitored at 256 nm and recorded on a chart recorder; 4 ml fractions were then collected. Flow rates of 60–80 ml/h were used for columns of Sephadex G-25 and 40–60 ml/h for columns of Sepharose 6B. The standard eluent used for all columns operated at room temperature contained 50 mM-Tris-HCl (pH 7.0), 100 mM-NaCl, 0.1% sodium dodecyl sulphate and 0.01% NaN_3 (to inhibit bacterial growth). When the columns were operated in the cold 0.1% sodium decyl sulphate was used in place of sodium dodecyl sulphate. In the work with Sephadex G-25 the inclusion of 0.1% Blue Dextran in the detergent used to stop the reaction enabled the void volume to be observed directly without using the spectrophotometer.

Both types of columns were used repeatedly without repouring. However, after a few months of continual use back pressure in the Sepharose 6B columns increased markedly and it was necessary to dismantle the column and soak the gel in 4 mM-imidazole-HCl

buffer, pH 7.0, for several weeks to wash out bound detergent.

Radioactivity in the column effluents was determined by measuring Čerenkov radiation in a liquid-scintillation spectrometer.

Polyacrylamide-gel electrophoresis. Standard equipment supplied by Shandon Southern Instruments Ltd., Camberley, Surrey, U.K., was used. The procedure was modified from one kindly communicated by Dr. E. M. Shooter (Stanford University, Stanford, Calif., U.S.A.), which employed a discontinuous buffer system. The upper electrode buffer (pH 8.7) contained 53 mM-Tris base, 50 mM-glycine and 0.1% sodium dodecyl sulphate; 2-mercaptoethanol to a concentration of 0.01% was added just before use. The lower electrode buffer contained 100 mM-Tris-HCl (pH 8.1). The sample buffer (pH 8.7) contained 53 mM-Tris base, 50 mM-glycine, 1% (w/v) sodium dodecyl sulphate, 5 M-urea, 10% (w/v) sucrose and 0.01% 2-mercaptoethanol (added before use). The mixture for 10% gels consisted of 0.99 g of acrylamide, 10 mg of *NN'*-methylenebisacrylamide, 3 g of urea (final concentration 5 M), 0.6 ml of riboflavin 5-phosphate solution (0.1 mg/ml, freshly prepared), 0.01% mercaptoethanol and 0.1% sodium dodecyl sulphate made up to 10 ml with 0.37 M-Tris-HCl (pH 8.7). For 15% gels, 1.49 g of acrylamide and 10 mg of *NN'*-methylenebisacrylamide were taken. The gels were photopolymerized for 1 h by using a Photopol fluorescent lamp with 26 W daylight bulbs (Shandon Southern Instruments Ltd.). Protein samples from the columns were concentrated for electrophoresis by rapid filtration through Centriflo membrane cones (Amicon Co., Oosterhout, Netherlands). The cones were loaded with 6–7 ml of column effluent and centrifuged in a Mistral 6L centrifuge for 30 min at 1000 g. The film of protein remaining in the cones was taken up in a small volume of sample buffer so that 100–300 μg of protein was contained in 100 μl of buffer. Samples of 100 μl were applied to the gels. A starting current of 3 mA/tube was used, which declined to 1.5 mA/tube as the separation continued.

After electrophoresis the gels were removed from the tubes and stained with 1% (w/v) Naphthalene Black in methanol–water–glycerol–acetic acid (50:50:20:1, by vol.) for 30 min as described by Tombs (1965). Excess of dye was removed by rinsing with the same solvent mixture by rotation on a wheel at 30 rev./min.

The distribution of radioactivity in the gels was determined by freezing the gels with solid CO_2 and cutting 0.1 or 0.2 cm slices with a scalpel. The accuracy of the cutting was checked for each gel by measuring the length of the gel before and after slicing. Each slice was suspended in 5 ml of 0.5 M-NaOH in a scintillation vial and radioactivity determined by measurement of Čerenkov radiation. No increase in

efficiency was obtained by first homogenizing the slice in 0.5 M-NaOH or by solubilizing it with H_2O_2 before counting for radioactivity.

Labelling and determination of protein-bound serine phosphate. The reaction mixture contained 30 mM-Tris-HCl (pH 7.4), 5 mM- $[\text{}^{32}\text{P}]\text{ATP}$ (0.5×10^6 – 4.0×10^6 c.p.m./ μmol), 5 mM- MgSO_4 and 5 mg of membrane protein in a final volume of 2.5 ml. The reaction was stopped by the addition of 0.25 ml of 50% (w/v) sodium dodecyl sulphate. The solubilized membrane protein was fractionated by using a column of Sepharose 6B exactly as described above for material labelled with a low concentration of ATP.

To determine serine phosphate in the column effluents the procedure of Weller & Rodnight (1971) was applied to samples concentrated by ultrafiltration through Centriflo cones as described above.

Results

Nature of the sodium-dependent bound phosphate at neutral pH

Table 1 shows that the labelled protein formed in the Na^+ -dependent reaction with ATP had the same properties when isolated at neutral pH by gel filtration as when isolated by the usual acid-washing procedure (see Rodnight, 1970). The bound phosphate decreased to the control value after incubation for 2 min and did not accumulate in the presence of K^+ . The yield at room temperature was about 30% of that obtained under the acid conditions, the difference being due to loss of protein on the column and hydrolysis of the bond during elution (Alexander, 1971). Operating the columns at 2°C with sodium decyl sulphate as the denaturing and solubilizing agent resulted in a higher yield, presumably owing to a decrease in hydrolysis during elution; however, since a quantitative yield was not essential in the present study, elution was normally done at room temperature with the more readily available dodecyl salt.

In the above experiments the protein concentration in the reaction mixture was 0.2 mg/ml and the detergent/protein ratio (in mg) used to stop the reaction was 5:1 (procedure A). In experiments where the higher protein concentration (2–4 mg/ml) and a detergent/protein ratio of 18:1 were used (procedure B), the yield of bound phosphate at room temperature was always higher than those values observed in the experiments with procedure A, amounting to around 62 pmol/mg of protein. When corrected for losses of protein and label on the column a value of 130 pmol/mg of protein was obtained, which compares well with the yield obtained in previous work by using acid precipitation (Rodnight, 1970). The more satisfactory yield in procedure B was probably due to the very short period of labelling and to the more rapid denaturation achieved when the higher detergent/protein ratio was used.

Table 1. *Gel filtration of labelled membrane protein on Sephadex G-25*

Membrane protein (0.5 mg) was labelled by procedure A. The reaction was stopped with 0.25 ml of 1% sodium dodecyl sulphate for column operation at room temperature (18–24°C) and 0.25 ml of sodium decyl sulphate for operation at 2°C. Labelled protein was isolated on Sephadex G-25 as described in the Experimental section. The eluent used for columns operated at room temperature contained 0.1% sodium dodecyl sulphate and at 2°C it contained 0.1% sodium decyl sulphate. The total void volume was collected and radioactivity determined by Čerenkov radiation. Results are means $\pm$ s.d. with the numbers of observations in parentheses.

Reaction conditions			Temperature of column (°C)	Bound phosphate recovered (pmol/mg of protein)		
Temperature (°C)	Na ⁺ (mM)	K ⁺ (mM)		at 0s	at 5s	at 120s
37	0	0	18–24	5.0 (3)	13.6 $\pm$ 2.2 (4)	—
37	50	0	18–24	—	48.4 $\pm$ 6.6 (5)	1.5 $\pm$ 0.2 (5)
37	50	1	18–24	6.7 $\pm$ 1.7 (4)	8.9 $\pm$ 3.1 (4)	—
37	50	0	2	—	70.6 $\pm$ 6.5 (5)	—
2	50	0	2	—	31.4, 35.8	—

Table 2. *Effect of hydroxylamine on the stability of the bound phosphate isolated at neutral pH values*

Membrane protein (7.9 mg) was labelled and isolated in 25 ml of eluent by procedure B. Bovine serum albumin [1 ml of a 1% (w/v) solution] was added to the 25 ml of eluate, followed by 20 ml of ice-cold acetone to each of four samples of 5 ml. After centrifuging at 2°C for 20 min at 2500g the precipitated protein was washed three times by resuspension and centrifuging with 4 ml of ice-cold water. The pellets were then suspended in 4 ml of water, and either 2.7 ml of 2M-hydroxylamine hydrochloride (at pH 7.0) or 2.7 ml of 2M-NH₄Cl was added. The suspensions were left at 0°C for 10 min, after which the protein was spun down and radioactivity in the pellets and supernatants determined. The results from the two distinct experiments are given.

Expt. no.	Treatment	Bound phosphate (pmol)		Amount released (%)
		Total	In pellet	
1	Hydroxylamine hydrochloride	50.9, 47.1	6.1, 5.9	88, 88
	NH ₄ Cl	45.3, 52.4	34.0, 28.0	25, 46
2	Hydroxylamine hydrochloride	58.0, 59.4	7.1, 4.9	88, 92
	NH ₄ Cl	59.5, 59.0	35.1, 32.1	41, 46

The lability of the bound phosphate to hydroxylamine under neutral conditions was studied by precipitating the labelled protein in the eluate by addition of cold acetone to a final concentration of 80% (v/v) and centrifuging for 20 min at 2500g. Addition of carrier protein (bovine serum albumin) was necessary to obtain full recovery of labelled protein. On suspension of the protein pellet in 0.8M-hydroxylamine-hydrochloride 90% of the bound radioactivity was released (Table 2), showing that the lability of the bond to hydroxylamine observed in previous work (Nagano *et al.*, 1967) was not due to exposure of the membrane protein to acid.

The influence of pH on the rate of spontaneous hydrolysis at 37°C of the bound phosphate in membrane protein isolated under neutral pH conditions was compared with that observed by Nagano *et al.* (1967) on acid-denatured protein (Fig. 1). The general shape of the curve for the detergent-treated protein was consistent with that expected for an acyl phosphate bond, although the bond was rather more stable than in the acid-treated protein, particularly at higher pH values. There was no evidence for an immediate

release of bound ³²P when the pH was altered to any of the values examined. Further, the percentage of ³²P released by hydroxylamine was independent of the time of exposure to acid conditions, confirming that no portion of the bound phosphate had undergone migration during the treatment at different pH values.

Fractionation of protein labelled by the Na⁺-dependent reaction on Sepharose 6B

In experiments designed to characterize further the protein labelled in the Na⁺-dependent reaction the solubilized membrane fragments were fractionated on columns of Sepharose 6B. The protein labelled in the presence of Na⁺ emerged between 80 and 110 ml with a peak at 95 ml (B) and was well separated from the labelled protein in the void volume (A); without added Na⁺ the major part of the label occurred in the void volume (Fig. 2). Approximately 25% of the applied protein was recovered in peak B, 10% in peak A and 20% in peak C. To obtain the maximum yield of Na⁺-dependent labelled protein in peak B it

Fig. 1. Stability of the bound phosphate isolated by gel filtration at different pH values

Microsomal protein (in batches of 7.9 mg) was labelled by procedure B and isolated in 25 ml of eluate on Sephadex G-25. Two control samples (1.0 ml) were treated with 0.25 ml of 100% (w/v) trichloroacetic acid, centrifuged immediately and the supernatant was separated. Four samples (5 ml) were then rapidly adjusted to each predetermined pH value with the minimum volume of 0.1 M-NaOH, 0.33 M- H_3PO_4 , acetic acid or 6 M-HCl. Immediately after adjustment of pH, and after 30 min at 37°C, samples (1 ml) were treated in duplicate with trichloroacetic acid as above and centrifuged. The radioactivity in the pellets and supernatants was determined and the percentage of the bound phosphate released was calculated. Each point is the mean of two to four distinct experiments. ○, Percentage of ^{32}P released from the protein isolated at neutral pH in sodium dodecyl sulphate; △, percentage of ^{32}P released from acid-denatured protein (Nagano *et al.*, 1967).

Fig. 2. Fractionation of labelled membrane protein on Sepharose 6B

Two samples of washed membrane protein (11 mg) were labelled by procedure B in the presence and absence of 50 mM-NaCl. The labelled protein was fractionated on identical columns of Sepharose 6B at a flow rate of 60 ml/h as described in the Experimental section. ---, Percentage transmission at 256 nm of the eluate; —, radioactivity in 4 ml fractions from sample labelled without Na^+ ; ···, radioactivity from sample labelled with Na^+ . For details of peaks A–C see the text.

was essential to maintain the detergent/protein ratio (in mg) in the sample applied to the column greater than 10:1.

The procedure gave a 3–5-fold purification of the Na^+ -dependent bound phosphate. Thus in a series of four experiments the bound phosphate in peak B amounted to an average of 53 pmol/mg of applied protein and 198 pmol/mg of peak protein. Correction of the latter value for hydrolysis on the column (estimated at 35%) gave a value of 300 pmol of bound phosphate/mg of protein. The ^{32}P present in peak B remained bound to the protein on precipitation with acetone and washing of the precipitate with water (see Table 2); 78% of the label was then released by exposure to 0.8 M-hydroxylamine. By contrast the label in peak A was almost entirely lost on precipitation and washing, suggesting it consisted of non-covalently bound ^{32}P . Treatment of labelled protein with hydroxylamine before separation on the column completely abolished the Na^+ -dependent labelling in peak B, but had little effect on the recovery of radioactivity in peak A.

The molecular weight of the protein labelled by the Na^+ -dependent reaction was determined by comparison of its elution volume with that of marker proteins determined on identical columns in the presence of sodium dodecyl sulphate (Table 3). The validity of this procedure is discussed by Alexander (1971). The markers used were bovine thyroglobulin (mol.wt. 670 000; Edelhoch, 1960), horse apoferritin (mol.wt. 480 000; Rothen, 1944) and jack-bean urease (mol.wt. 480 000; Sumner *et al.*, 1938). The ratio of the elution volume to the void volume was plotted against the logarithm of the molecular weight, a straight-line relationship being assumed. [This relationship was found to approach linearity for a wide range of proteins and gels (Whitaker, 1963; Leach & O'Shea, 1965), but we have not excluded the possibility that it departs from linearity for proteins of very high molecular weight.] By this procedure the molecular weight of the labelled protein prepared from ox brain was 570 000 with apoferritin as standard and 580 000 with urease. A single determination on membrane protein from guinea-pig cerebral cortex gave a value of 530 000.

Polyacrylamide-gel electrophoresis of protein labelled by the Na^+ -dependent reaction

Of the several procedures tried only the one described in the Experimental section gave useful separations. For rapid fractionation 10% gels were most satisfactory; labelled protein from peak B was separated into 12–15 bands in 40 min (see Fig. 5 for a typical run). Virtually all the Na^+ -dependent bound phosphate occurred in a single band from 1 to 3 cm from the origin (Fig. 3). Of the radioactivity

Table 3. *Estimation of the molecular weight of the labelled protein in peak B isolated on Sepharose 6B*

The void volume (V_0) was determined with Blue Dextran; V_e is the elution volume of the marker measured from the top of the protein peak. Marker proteins were dissolved in 0.1% sodium dodecyl sulphate and 100mM-NaCl and applied to the column in a volume of 2.75 ml; elution was continued with the same solution at a flow rate of 45 ml/h; other details of column operation are given in the legend to Fig. 2. Membrane protein was labelled by procedure B.

Markers	Weight of protein applied (mg)	V_e/V_0	log(Mol.wt.)	
Blue Dextran	—	1.08		
Thyroglobulin	10.0	1.08	5.826	
Apo ferritin	4.0	2.29	5.681	
Urease	10.0	2.44	5.681	
				log (Mol.wt.) estimated from above data
Labelled proteins				Estimated mol.wt.
From ox brain	10.0	1.57	5.755*	570000
			5.763†	580000
From guinea-pig brain	5.0	1.93	5.727*	530000
			5.735†	543000

* With apoferritin as lower mol.wt. marker.

† With urease as lower mol.wt. marker.

Fig. 3. *Fractionation of labelled protein from peak B on polyacrylamide gels*

Labelled protein from peak B of a Sepharose 6B column was concentrated by filtration on Centrifo membrane cones and fractionated on a 10% polyacrylamide gel as described in the Experimental section. The gel was stained with 1% Naphthalene Black, frozen with solid CO_2 and cut into 20 slices (0.1 cm) for the determination of the distribution of radioactivity by the procedure given in the Experimental section. The figure shows the first 2 cm of the gel and the position of the band labelled by the Na^+ -dependent reaction. The filled area indicates the radioactivity remaining in the band after treatment with 0.8M-hydroxylamine hydrochloride. Electrophoresis was continued for 40 min.

present in this band 70–88% was released by 0.8M-hydroxylamine (four observations), as compared with 5–14% by the same concentration of NH_4Cl . However, owing to the rapid hydrolysis of the acyl phosphate bond at the pH (8.7) of the sample buffer the recovery of radioactivity in the band was only about 1–2%. A more complete fractionation of the components of peak B was achieved by electrophoresis on 15% gels for 75 min, which gave up to 18 bands. Under these conditions also, the Na^+ -dependent labelling was confined to a single band.

Further evidence for the homogeneity of the labelled band was obtained as follows. (1) The band from a 15% gel was isolated as a 0.2 cm section and layered on another 15% gel; a single band running in the same position resulted after electrophoresis. (2) The same procedure was repeated except the second gel contained 8M- instead of 5M-urea; again a single band resulted. (3) The band was isolated as above and run for a second time on a 15% gel by using an upper buffer of pH 10 and a lower buffer of pH 8.8 and the gel mixture buffered at pH 10 with a Na_2CO_3 – NaHCO_3 buffer containing Cl^- . After electrophoresis for 75 min in this system a single band was obtained which migrated at a slower rate than in the original system.

It was also just possible to detect Na^+ -dependent bound phosphate in a single band on 15% gels without prior fractionation of the labelled protein on Sepharose 6B. The Na^+ -dependent labelling occurred in the same position as in peak B material, but without the Sepharose 6B step another labelled band ran very close to it, making it difficult to interpret the results.

The molecular weight of the protein containing the Na^+ -dependent label was determined by comparison

of its rate of migration in the gel with marker proteins of known molecular weight. The work of Shapiro *et al.* (1967), Weber & Osborn (1969) and others has established a linear relationship between the mobility of a wide range of proteins in polyacrylamide gels containing sodium dodecyl sulphate and the logarithm of their molecular weights. The three proteins used in the present work as markers were lactate dehydrogenase (Castellino & Barker, 1968; mol.wt. 140000), hexokinase (Berger *et al.*, 1946; mol.wt. 96600) and pyruvate kinase (Steinmetz & Deal, 1966; mol.wt. 57000), which had mobilities in 10% gels of 0.11, 0.26 and 0.34 respectively. Lactate dehydrogenase is dissociated into its component subunits on exposure to sodium dodecyl sulphate (Di Sabato & Kaplan, 1964), but there was enough undissociated enzyme present in the short running time of our experiments to enable its use as a marker. Under identical conditions of electrophoresis the mobility of the labelled protein was 0.23, and a molecular weight of 102000 was determined graphically. It appears therefore that there was a considerable further dissociation of the protein on exposure to urea at the pH (8.7) of the buffer used in the gels.

Fractionation of membrane protein labelled by protein kinase activity in the absence of Na^+

The aim of these experiments was to investigate the possibility that a component of the Na^+, K^+ -ATPase contains serine hydroxyl groups that are phosphorylated by ATP in a reaction stimulated by cyclic AMP. For this purpose it was necessary to phosphorylate in the absence of Na^+ with a high concentration of ATP and to allow the reaction to proceed for at least 1 min, in order to obtain sufficient label in the serine residues. Under these conditions three peaks of radioactivity (Fig. 4, labelled peaks A', B' and C') were observed on fractionation on Sepharose 6B, corresponding to peaks A, B and C seen in the other experiments. However, a high percentage of the label in column eluate was lost during concentration by ultrafiltration (see below), although in all three peaks the residual label was stable to hydroxylamine treatment. The position of the hydroxylamine-stable label in peak B' was identical with the Na^+ -dependent labelling of peak B determined in parallel in a series of nine experiments. Cyclic AMP stimulated labelling mainly in peak B' (Fig. 4; Table 4).

As mentioned above, of the radioactivity present in the three peaks only 3–15% was recovered, after concentration of the eluate, as labelled serine phosphate by using the method of Weller & Rodnight (1971). Up to 23% of the radioactivity was lost in the filtrate from the Centriflo cones and this fraction was at first considered to consist of ^{32}P non-covalently bound to protein or lipid. However, substitution of

$^{32}\text{P}_i$ for $[\text{P}^{32}]\text{ATP}$ in the reaction mixture did not result in any significant labelling of protein. Binding of $[\text{C}^{14}]\text{ATP}$ was also low, and the binding of $^{32}\text{P}_i$ and $[\text{C}^{14}]\text{ATP}$ taken together only accounted for 10% of the total label in the eluate. About 25% of labelled protein was also lost through binding to the filtration cones, but the sum total of the losses from all sources only explained 50% of the difference between the radioactivity in the column eluate and that in serine phosphate isolated by paper electrophoresis. It seems likely therefore that some covalent binding of ^{32}P to other components in the membrane protein had occurred. This is supported by the observation that the percentage increase in serine phosphate labelling by cyclic AMP in peaks A' and B' was much greater than the increase in total labelling (Table 4).

Polyacrylamide-gel electrophoresis of protein in peak B'. When protein was labelled for 2 min with 5 mM- $[\text{P}^{32}]\text{ATP}$, with and without cyclic AMP, fractionated on Sepharose 6B and peak B' examined by gel electrophoresis, most of the label appeared to occur in two major fast-running bands (Fig. 5). The stimulating effect of cyclic AMP also occurred in the area of these major bands and no stimulation of labelling was observed in the band corresponding in position to the Na^+ -dependent label. The major bands contained some lipid, since they stained with 1% (w/v) Sudan Black, but the label was unlikely to be associated with lipid, since Weller (1969) showed that no significant labelling of lipid occurred under the conditions of labelling used here. The molecular weights of the materials in the two major bands were less than 45000.

Fig. 4. Fractionation of hydroxylamine-stable labelled protein on Sepharose 6B

Membrane protein (5mg) was labelled for 2 min with 5 mM- $[\text{P}^{32}]\text{ATP}$, solubilized with sodium dodecyl sulphate and fractionated on Sepharose 6B. —, Percentage transmission at 256nm of eluate; ····, radioactivity in eluate after labelling in the presence of 100 μM -cyclic AMP; —, radioactivity in eluate after labelling in the absence of cyclic AMP.

Table 4. *Stimulation of hydroxylamine-stable labelling by cyclic AMP*

Membrane protein (5 mg) was labelled with [32 P]ATP in the presence and the absence of 100 μ M-cyclic AMP for 2 min at 37°C and fractionated on Sepharose 6B as described in the Experimental section. After concentration of the eluate by ultra-filtration on Centrifo cones, serine phosphate was isolated and its radioactivity determined by the method of Weller & Rodnight (1971). The recovery of serine [32 P]phosphate is expressed as the percentage of the total radioactivity in the peaks. Serine phosphate was determined in only one experiment.

Peak	Recovery of serine [32 P]phosphate (%)	Stimulation of labelling in peaks by cyclic AMP (%)	Stimulation of serine phosphate labelling by cyclic AMP (%)
A'	3	31, 40	70
B'	4	71, 71	291
C'	15	17, 28	0

Fig. 5. Polyacrylamide-gel electrophoresis of hydroxylamine-stable labelled protein

Membrane protein (5 mg) was labelled for 2 min with and without cyclic AMP under the conditions indicated in Fig. 4 and the Experimental section. After fractionation on columns of Sepharose 6B, peak B' was concentrated and further fractionated on 10% polyacrylamide gels. The conditions of electrophoresis and the method of determining the radioactivity profile are given in Fig. 3 and the Experimental section. —, Radioactivity profile for sample labelled in the absence of cyclic AMP; ----, radioactivity profile for sample labelled in the presence of 100 μ M-cyclic AMP. The arrow indicates the position of the band labelled by the Na⁺-dependent reaction.

Labelling of membrane protein with di-isopropyl [32 P]phosphorofluoridate. The specificity of the inhibition of Na⁺,K⁺-ATPase activity by high concentrations of di-isopropyl phosphorofluoridate (Hokin & Yoda, 1964) has been questioned (Yoshida *et al.*, 1968; Lahiri & Wilson, 1971), but since other serine-reacting reagents have been found to inhibit the enzyme (Sachs *et al.*, 1969) the specific phosphorylation of a serine residue in the enzyme by the fluoridate cannot be excluded. To determine whether the re-

agent was bound to the protein containing the Na⁺-dependent bound phosphate, 6 mg of protein was incubated for 15 min at 37°C with 10 mM-di-isopropyl [32 P]phosphorofluoridate in 75 mM-Tris-HCl (pH 7.7) and fractionated on Sepharose 6B. The main peak of labelled protein occurred in an intermediate position between peaks B' and C', giving a molecular weight of about 520 000. Some labelled protein, however, occurred at the elution volume of peak B. This was concentrated on Centrifo cones and fractionated on 15% polyacrylamide gels. Two labelled bands were obtained, one of which was identical in position with the band labelled by the Na⁺-dependent reaction with [32 P]ATP.

Previous work with our system showed that in the presence of 3 mM-ATP inhibition of the enzyme by 3 mM-di-isopropyl phosphorofluoridate is completely reversed (Alexander, 1971). Protein (6 mg) was therefore labelled with the [32 P]phosphorofluoridate in the presence and the absence of 3 mM-ATP, and then fractionated on identical columns of Sepharose 6B. No difference could be detected in the profile of radioactivity in the two column eluates. However, when protein from the position of peak B was concentrated and fractionated on 15% polyacrylamide gels, the labelling of the band of approximate molecular weight 102 000 (i.e. that corresponding to the Na⁺-dependent labelling) was decreased in the presence of ATP by 53 and 57% in two distinct experiments. Protection by ATP was presumably not detectable on the column because of the low radioactivity present in the position of peak B, compared with the adjacent large peak of labelling.

Discussion

Nature of the Na⁺-dependent labelling at neutral pH

The results strongly suggest that there is no essential difference between the Na⁺-dependent bound phosphate stabilized by sodium dodecyl sulphate at neutral pH and that stabilized by strong acid. The dependence of phosphate transfer on the presence of Na⁺, and the lability of bound phosphate to the presence of K⁺ (Table 1), indicate the involvement of the

protein-bound phosphate in the Na^+, K^+ -ATPase. The influence of pH on the rate of hydrolysis of the bound phosphate, and its rapid release on exposure to hydroxylamine, are both consistent with an acyl phosphate bond. The reactivation by hydroxylamine of certain esterases such as chymotrypsin after inhibition by phosphorylation of active serine residues (Cunningham, 1954) is a very much slower and less complete process than the hydroxylamine-catalysed release of the Na^+ -dependent bound phosphate. The fact that the detergent-treated bound phosphate was more stable under alkaline conditions to hydrolysis than was the acid-treated material (Fig. 1) is probably due to protection of the acyl phosphate bond by strong hydrophobic binding of the sodium dodecyl sulphate carbon chain to protein.

It is noteworthy that in the absence of Na^+ most of the hydroxylamine-stable labelling was found in peak A on the Sepharose 6B column (Fig. 2), suggesting that the membrane fragments contain at least two enzyme systems competing for ATP, the Na^+, K^+ -ATPase and intrinsic protein kinase(s). The present results indicate that this hydroxylamine-stable component of labelling is due to the phosphorylation of serine residues in protein unconnected with the Na^+, K^+ -ATPase.

Nature of protein-bound phosphate isolated on Sepharose 6B

There is some evidence to suggest that the phosphorylated protein isolated on the Sepharose 6B column after procedure B is the protein of the complete Na^+, K^+ -ATPase system. Estimates of the molecular weight of this enzyme have varied widely from about 250000 to 700000, discrepancies probably being due to the widely differing purification methods, and to uncertainty about some of the techniques involved (Kepner & Macey, 1968, 1969; Kahlenberg *et al.*, 1969). Brain Na^+, K^+ -ATPase preparations have been estimated to have a molecular weight of about 500000 by the radiation-inactivation technique (Nakao *et al.*, 1967) and by sucrose-density-gradient centrifugation (Mizuno *et al.*, 1968). The molecular weight of a Lubrol-solubilized Na^+, K^+ -ATPase fraction from bovine brain microsomal fraction was reported as 560000 by column chromatography; however, this differs widely from an estimate of 250000 for a Lubrol-solubilized preparation from pig brain (Atkinson *et al.*, 1971). It seems reasonable therefore to suggest that the phosphorylated component of molecular weight 570000–580000 reported here probably represents the Na^+, K^+ -ATPase protein. If it only represents one component of the enzyme, then the system as a whole would have a much higher molecular weight than the estimates of other workers referred to above. That the enzyme is not dissociated into its component subunits on isolation on Sepharose

6B is also supported by the fact that other high-molecular-weight proteins, such as thyroglobulin and ureas, were not dissociated under identical conditions, though their component subunits were obtained on fractionation by polyacrylamide-gel electrophoresis under the conditions described in the present paper (D. R. Alexander, unpublished work).

Isolation of a phosphorylated subunit

The protein of molecular weight 570000–580000 isolated on the Sepharose 6B column was dissociated on exposure to 5M-urea, alkaline pH and a sodium dodecyl sulphate concentration ten times that of the column eluent. At least one of these subunits has an apparent molecular weight of 102000 (Fig. 3a). Since the initial publication of this work (Alexander & Rodnight, 1970), several studies have been published on the subunit structure of the Na^+, K^+ -ATPase which are in good agreement with the work described here. Uesugi *et al.* (1971) isolated a phosphorylated subunit of molecular weight 94000 from bovine brain microsomal fraction, and Atkinson *et al.* (1971) have obtained a phosphorylated subunit of molecular weight 98000–100000 from pig brain microsomal fraction, though in the latter case the subunit could be further dissociated into smaller subunits. Phosphorylated components have also been obtained from brain membrane fragments prepared from seven different species, ranging in molecular weight from 89000 to 95000 (Collins & Albers, 1972), and also from erythrocyte membrane protein (mol.wt. 98000) (Williams, 1972). A major component of molecular weight 84000 occurs in a Na^+, K^+ -ATPase preparation from canine renal medulla (Kyte, 1971). It is not yet possible to say from the work published here whether other subunits may be involved in the structure of the enzyme, nor how many subunits of molecular weight 102000 are present.

Hydroxylamine-stable phosphate labelling

There is no evidence in the present work for any direct connexion between the Na^+, K^+ -ATPase and intrinsic protein kinase activity in the membrane fragments. No hydroxylamine-stable labelling was found in the subunit of molecular weight 102000 (Fig. 5), though the possibility has not been rigorously excluded that the two labelled bands of lower molecular weight are connected with the intact ATPase enzyme. That the protein kinase activity is unconnected with the Na^+, K^+ -ATPase is also suggested by the fact that 3mM-di-isopropyl phosphorofluoridate inhibited the Na^+, K^+ -ATPase by 70%, whereas it had no effect on the protein kinase activity, although 1mM- MnCl_2 inhibited the kinase activity by 25% and yet had no effect on the Na^+, K^+ -ATPase (Alexander, 1971). Further, cyclic AMP has no effect on the

Na⁺,K⁺-ATPase preparation used in the present work (R. Rodnight & D. Carrera, unpublished work).

Reaction with di-isopropyl phosphorofluoridate

The finding that di-isopropyl [³²P]phosphorofluoridate was present in the subunit of molecular weight 102000, and that this labelling was decreased by 55% by the presence of 3mM-ATP in the reaction mixture, suggests that a serine residue is involved at the active centre of the enzyme, and that it may be involved in the binding of ATP to the enzyme before phosphorylation. The fact that most of the di-isopropyl [³²P]phosphorofluoridate labelling was found in proteins unassociated with the Na⁺,K⁺-ATPase casts doubt on experiments where fractionation of protein with di-isopropyl [³²P]phosphorofluoridate was not carried out before polyacrylamide-gel electrophoresis. Our work therefore agrees with that of Chignell & Titus (1969) in that the band phosphorylated by ATP in a Na⁺-dependent reaction also contains a di-isopropyl phosphorofluoridate-reactive serine residue, but suggests that the second band containing di-isopropyl [³²P]phosphorofluoridate found by these workers represents protein unconnected with the Na⁺,K⁺-ATPase. The work reported here therefore does not support the suggestion that two separate proteins may be involved in the Na⁺-dependent phosphorylation (Chignell & Titus, 1969).

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