Chemical Modification of a Cerebral Sodium-plus-Potassium Ion-Stimulated Adenosine Triphosphatase Preparation

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(Received 1 May 1970)

1. A particulate $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase preparation obtained by treatment of bovine cerebral microsomes with a sodium iodide reagent has been further treated with acid anhydrides likely to convert amino groups into acidic derivatives. 2. The extent of acylation of amino groups was determined by reaction of the remaining amino groups with 2,4,6-trinitrobenzenesulphonic acid. The unmodified preparation contains about $1.2 \mu\text{equiv.}$ of amino groups/mg of protein of which only about $0.5 \mu\text{equiv.}$ are accounted for by protein amino groups. Kinetics of the trinitrobenzenesulphonic acid reaction with the unmodified preparation are complex and are altered by ATP or ouabain. 3. The compounds examined cause loss of $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase activity when relatively few amino groups are modified but ATP was found to afford partial protection against inactivation by methylmaleic anhydride. $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase activity is partly restored to the dimethylmaleylated preparation by hydrolysis of the dimethylmaleyl-amide bonds but not if more than about 20% of the amino groups have been acylated. 4. Supernatants obtained by high-speed centrifugation of the dimethylmaleylated preparation contained up to 45% of the total protein with less than 10% of the total phospholipid. Methylmaleyl and benzenetricarboxyl derivatives of the enzyme preparation behaved similarly but tetrafluorosuccinylated material was almost entirely deposited by centrifugation.

The enriched Na,K-ATPase^* preparation obtained by treatment of cerebral microsomes with a sodium iodide reagent affords scope for further separation of enzymically active from inactive protein since estimates suggest that only about 10% of the protein could account for the observed Na,K-ATPase activity (Kepner & Macey, 1969; Kahlenberg, Dulak, Dixon, Galsworthy & Hokin, 1969). Sodium iodide-treated material from bovine cerebral microsomes is also enriched in lipid constituents (Pull & McIlwain, 1970) so that mild procedures separating lipid from protein will also be valuable in identifying the molecules necessary for Na,K-ATPase activity and particularly may clarify the role of phospholipid which appears essential for this enzyme (Wheeler & Whittam, 1970). Detergents have commonly been used to solubilize and further enrich the cerebral microsomal Na,K-ATPase (Schoner, von Ilberg, Kramer & Seubert, 1967; Medzihradsky, Kline & Hokin, 1967) but appreciable proportions of lipid constituents are also

solubilized (Swanson, Bradford & McIlwain, 1964). Considerations such as these have prompted attempts to dissociate the sodium iodide-treated bovine cerebral microsomal Na,K-ATPase preparation by chemical modifications that may be reversed under mild conditions.

Conversion of amino groups into acidic derivatives by reaction with dicarboxylic acid anhydrides was considered likely to disrupt the enzyme preparation since succinylation of a water-soluble myelin protein prevents it from forming insoluble complexes with anionic lipids (Braun & Radin, 1969). Additionally, some normally insoluble proteins such as myosin (Oppenheimer, Barany, Hamoir & Fenton, 1967), mitochondrial structural protein (MacLennan, Tzagoloff & Rieske, 1965) and the apoprotein of human serum β -lipoprotein (Scanu, Pollard & Reader, 1968) become soluble after reaction with succinic anhydride. Rabbit muscle aldolase is dissociated by maleylation and removal of the maleyl groups by mild acid hydrolysis restores a considerable proportion of enzyme activity (Sia & Horecker, 1968).

Succinyl-amide bonds withstand hydrolysis of

* Abbreviations: Na,K-ATPase , $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

proteins and conditions for removal of maleyl groups were also considered too harsh for Na,K-ATPase activity to survive. The acyl-amide bonds formed by tetrafluorosuccinic anhydride (Braunitzer, Beyreuther, Fujiki & Schrank, 1968), methylmaleic anhydride and dimethylmaleic anhydride (Dixon & Perham, 1968) are more labile so that these compounds and also benzenetricarboxylic anhydride have been examined for effects on the Na,K-ATPase preparation.

EXPERIMENTAL

Chemicals. Tetrafluorosuccinic anhydride, prepared by Peninsular Chemresearch Inc., was supplied by Kodak Ltd., Kirkby, Lancs., U.K. 2,3-Dimethylmaleic anhydride and 1,2,4-benzenetricarboxylic anhydride were from R. N. Emanuel Ltd., Alpertton, Middlesex, U.K. The 2,4,6-trinitrobenzenesulphonic acid used for amino group determinations, *p*-nitrophenol, disodium-*p*-nitrophenolphosphate and ATP (disodium salt) were obtained from the Sigma (London) Chemical Co. Ltd., London, S.W.6, U.K. Crystalline bovine serum albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. Crystalline 2,4,6-trinitrobenzenesulphonic acid tetrahydrate was used for the kinetic experiments and was obtained, together with other chemicals that were of the highest available grade, from British Drug Houses, Ltd., Poole, Dorset, U.K. Solutions of ATP (disodium salt) and disodium-*p*-nitrophenyl phosphate were deionized and neutralized with tris, for use in enzyme assays, or KOH for other additions.

Enzyme preparation and assays. The procedures for obtaining an enriched Na,K-ATPase preparation by treatment of bovine cerebral microsomes with a NaI reagent (Nakao, Tashima, Nagano & Nakao, 1965) and assay of Na,K-ATPase activity were as described by Pull & McIlwain (1970). Media for assay of *p*-nitrophenylphosphatase activity contained 3mM-MgCl₂, 3mM-*p*-nitrophenyl phosphate, 50mM-tris-hydrochloric acid buffer, pH7.4, with or without 30mM-KCl in a final volume of 1ml. The reaction was stopped after 10min at 37°C by the addition of 1ml of 6% (w/v) trichloroacetic acid followed by 0.5ml of 2M-tris and 1ml of water. The extinctions of the supernatants obtained after centrifugation were determined at 420nm together with those given by known amounts of *p*-nitrophenol.

Chemical determinations. Amino groups of the Na,K-ATPase preparation and of its acyl derivatives were determined with TNBS by the method of Habeeb (1966) except that the reaction time was shortened to 1h. Before the determination of thiol groups with 5,5'-dithiobis-(2-nitrobenzoic acid) as described by Ellman (1959), the samples were solubilized with a final concentration of 1% (w/v) sodium dodecyl sulphate containing 50mM-sodium phosphate buffer, pH8.0. This concentration of sodium dodecyl sulphate does not interfere with the reaction (Criddle, Edwards & Petersen, 1966). The method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Miller (1959) was used for protein determinations with crystalline bovine serum albumin as standard. Phospholipids were extracted with chloroform-methanol (2:1, v/v), digested with HClO₄ and P_i was determined as previously described (Pull & McIlwain, 1970).

Treatment with acid anhydrides. The enzyme preparation could be treated with small amounts of the anhydrides at constant pH by using a concentrated *N*-tris(hydroxymethyl)methylglycine buffer solution, pH8.0 (adjusted with KOH). Several experiments were carried out in this way and the results were essentially the same as those obtained by using the following pH-stat procedure which was found to be more suitable.

Reactions were carried out in the titration cell of a Radiometer pH-stat assembly with magnetic stirring and at room temperature except for tetrafluorosuccinylation which was at 5°C. The enzyme preparation contained about 5mg of protein in 1.5 or 2ml of 3mM-sodium tetraborate (pH8.0 or 8.5 with boric acid) or 50mM-sodium cacodylate (pH7.0 with HCl). After addition of the anhydride the chosen pH was maintained within 0.1 pH unit by automatic additions of a standardized KOH solution of appropriate strength, usually 0.5 or 1.0M. A single addition of the anhydride was made except for tetrafluorosuccinic anhydride which was added in approx. 1μl volumes, each addition being allowed to react fully before the next. The amount of anhydride added was taken to be that calculated from the total KOH consumed. When base uptake had ceased the reaction products were diluted to 5ml and 2ml was centrifuged at 150000g for 1h in a Spinco L2-65 centrifuge. Samples were kept cold and all operations and analyses were performed within 3-4h after acylation, except in specified experiments that tested for restoration of amino groups to the modified material.

Additions that would interfere with subsequent assays and analyses were removed by dialysing the samples against 5mM-*N*-tris(hydroxymethyl)methylglycine buffer adjusted to pH8.0 with KOH. This buffer was chosen since it did not react with TNBS or significantly alter the cation composition of enzyme assay media when the dialysed sample was added.

RESULTS

2,4,6-Trinitrobenzenesulphonic acid. This reagent was used for the determination of amino groups in the Na,K-ATPase enzyme preparation since it reacts favourably with amino groups under mild alkaline conditions (Okuyama & Satake, 1960), and though *S*-trinitrophenyl derivatives can form they are unstable above pH 8 (Kotaki, Harada & Yagi, 1964). Reaction of the enzyme preparation with TNBS at pH8.5 and 40°C was complete after 1h and the extinction at 340nm of the trinitrophenylated material, after acidification and solubilization with sodium dodecyl sulphate, was directly proportional to the amount of sample up to a protein content of 0.4mg. The amino groups of bovine serum albumin in this procedure gave trinitrophenyl derivatives with ϵ 0.995 × 10⁴ (Habeeb, 1966) and this value has been assumed for the trinitrophenyl-amino groups of the Na,K-ATPase preparation.

The enzyme preparations were found in this way to contain between 1.1 and 1.3μequiv. of amino groups/mg of protein. Additions were made of

Fig. 1. Trinitrophenylation of the enzyme preparation in the presence of ATP or ouabain at 40°C. (a) Reaction was between enzyme preparation containing 0.3 μ equiv. of amino groups and 3.4 μ mol of TNBS in 3 ml of 0.16 M-KHCO₃ (pH 8 with KOH) with 1 mM-ATP (●) or without ATP (○). In (b) 0.23 μ equiv. of amino groups was present, NaHCO₃ at pH 8.5 (with NaOH) was used and ouabain was absent (○) or at a concentration of 33 μ M (●). The increase in extinction of the reaction mixtures at 335 nm was determined at intervals against similar mixtures, containing no enzyme preparation, in a Unicam SP.800 recording spectrophotometer until there was no further change.

agents that modify Na,K-ATPase activity and that were considered likely to alter the enzyme preparation so as to reveal any masked amino groups. No differences were found in the number of amino groups measured when the sample included 3 mM-ATP (potassium salt), 3 mM-ATP (potassium salt) + 5 mM-MgCl₂, 10 μ M-ouabain or 0.1% (w/v) of the non-ionic detergent Lubrol W; the amino group of ATP did not react with TNBS. Precipitation of the enzyme preparation with trichloroacetic acid slightly decreased the number of amino groups reacting with TNBS. Extraction of the trichloroacetic acid precipitate with ethanol-ether (1:1, v/v), chloroform-methanol (2:1, v/v) and ether removed about 58% of the TNBS-reactive groups. The protein residue, after dissolving in sodium hydroxide and neutralizing with hydrochloric acid, accounted for 0.48 μ equiv. of amino groups/mg of protein out of a total in the unextracted precipitate of 1.15 μ equiv./mg of protein.

The reaction of the enzyme preparation with TNBS was also followed spectrophotometrically until there was no further increase in extinction at 335 nm and results are expressed semi-logarithmically in Fig. 1. Assuming all of the trinitrophenyl-amino groups contribute equally to the measured extinction it is apparent that the reaction kinetics under these conditions are complex but appear to comprise an initial reaction of two sets of amino groups, at different rates, followed by rapid reaction of a third set. The acceleration of the reaction when about 85% of the amino groups have been trinitrophenylated suggests a conformational change that exposes further amino groups to the reagent. A similar acceleration effect occurs in the reaction of lysozyme with TNBS (Freedman & Radda, 1968). The initial rate of trinitrophenylation of the Na,K-ATPase preparation was increased by including ATP in the reaction (Fig. 1). Enzyme hydrolysis of the added ATP was prevented

Table 1. *Enzyme activities after treatment with methylmaleic anhydride*

Samples of the enzyme preparation containing 4 mg of protein in 1.5 ml of 3 mM-sodium borate buffer, pH 8, were treated with increasing amounts of methylmaleic anhydride, dissolved in dioxan at room temperature. The pH was maintained constant by pH-stat addition of 0.25 M-KOH. The concentration of added dioxan was 0.3% (v/v) in the control and all other samples during acylation and was 0.005% during enzyme assays.

	Methylmaleic anhydride (mol/equiv. of amino groups)					
	0	0.42	0.84	1.26	1.68	2.10
Remaining amino groups (μ equiv.)	5.2	4.8	4.5	4.1	3.7	3.5
Adenosine triphosphatase: (μ mol/h per mg of protein)						
With Na ⁺	60.0	15.4	8.1	4.3	3.8	1.6
Without Na ⁺	1.9	0.5	1.0	1.1	2.0	1.5
<i>p</i> -Nitrophenol phosphatase: (μ mol/h per mg of protein)						
With K ⁺	8.3	4.1	2.8	1.8	1.3	1.2
Without K ⁺	0.7	0.8	0.9	0.7	0.8	0.6

by omitting Mg²⁺ but ADP and P_i will accumulate fairly rapidly at 40°C. The inclusion of ouabain, which inhibits Na,K-ATPase activity of the sodium iodide-treated preparation (Nakao *et al.* 1965) does not alter the initial rate but diminishes it at a later stage (Fig. 1).

2-Methylmaleic anhydride. The K⁺-stimulated *p*-nitrophenol phosphatase activity associated with Na,K-ATPase preparations has been suggested to represent an intermediate stage in the overall Na,K-ATPase reaction sequence (Ahmed & Judah, 1964). Both of these enzymes were inactivated by reaction with methylmaleic anhydride whereas the low concentrations of Na⁺-independent adenosine triphosphatase and K⁺-independent *p*-nitrophenol phosphatase, which this preparation contains, were not altered (Table 1). The Na,K-ATPase is 50% inactivated when 4% of the amino groups have been modified but the K⁺-stimulated *p*-nitrophenol phosphatase is slightly less sensitive to modification of amino groups.

Reaction of the enzyme preparation at a protein concentration of 3 mg/ml with a fourfold molar excess of methylmaleic anhydride at pH 8 caused 50% of the amino groups to be acylated. When 3 mM-ATP was included in the reaction a 2.5-fold molar excess of the anhydride sufficed to acylate 50% of the amino groups and inactivation of the Na,K-ATPase was decreased. There was no loss of Na,K-ATPase activity when 10% of the amino groups were modified and a 50% inactivation of the enzyme occurred when 24% of the amino groups were acylated. An experiment comparing 3 mM-ATP and 3 mM-AMP with a single concentration of methylmaleic anhydride showed AMP to be without effect either in increasing acylation of the preparation or decreasing loss of Na,K-ATPase activity.

Suspensions of the preparation are less turbid

Methylmaleic anhydride (mol/equiv. of amino groups)

Fig. 2. Changes in the Na,K-ATPase preparation after reaction with methylmaleic anhydride at pH 8.0 in a pH-stat with additions of 0.5 M-KOH. Protein (■) and lipid phosphorus (□) in the supernatant of centrifuged samples are shown in (a) whereas (b) shows the changes in amino groups (●), thiol groups (△), measured protein content (■), *E*₄₅₀ (□) and Na,K-ATPase activity (○). Results are expressed as percentages of material to which no anhydride was added but which was otherwise treated similarly and had 4.6 mg of protein, 5 μ mol of lipid phosphorus, 5.7 μ equiv. of NH₂, 120 n-equiv. of SH groups, an extinction at 450 nm of 1.122 and Na,K-ATPase activity of 61 μ mol of P_i/h per mg of protein.

after reaction with methylmaleic anhydride and the measured content of protein and thiol groups is slightly altered (Fig. 2). Methylmaleic anhydride

was considered likely to react only with amino groups since maleic anhydride was found to be specific for the amino groups of several proteins (Butler, Harris, Hartley & Leberman, 1969). The changes in protein and thiol groups of the preparation were attributed to conformational changes, consequent on methylmaleylation, exposing further thiol groups and masking tyrosine residues. Maleic anhydride has since been reported capable of reacting with protein groups other than amino (Gibbons & Perham, 1970) so it is possible that the hydroxyl group of tyrosine residues in the Na,K-ATPase preparation is being esterified with methylmaleic anhydride. Higher concentrations of methylmaleic anhydride acylate up to 90% of the amino groups and there is complete loss of Na,K-ATPase activity at these degrees of modification. Centrifugation of the acylated material gives clear supernatants that contain up to 30% of the total protein with less than 5% of the total lipid phosphorus (Fig. 2). No Na,K-ATPase activity is found in any of the supernatants and Na,K-ATPase activity remaining after methylmaleylation is recovered in the deposit.

Attempts to restore Na,K-ATPase activity to the acylated material by acid hydrolysis of the methylmaleyl-amide bonds were not successful. Na,K-ATPase activity of the unmodified enzyme preparation was found to be largely lost below pH 6 and regeneration of amino groups to the methylmaleylated material proceeded very slowly at this pH.

2,3-Dimethylmaleic anhydride. Dimethylmaleyl-amide bonds are significantly hydrolysed over several hours at pH 8 (Dixon & Perham, 1968) so reaction of the Na,K-ATPase preparation with dimethylmaleic anhydride was performed at pH 8.5 which was also found to be the optimum pH for acylation of the amino groups. No hydrolysis of dimethylmaleyl-amide bonds appeared to take place during the determination of free amino groups with TNBS, judged by measuring the extent of trinitrophenylation of acylated material at time-intervals up to 1 h.

The correspondence between loss of Na,K-ATPase activity and loss of amino groups after reaction of the enzyme preparation with dimethylmaleic anhydride is similar to that given by methylmaleic anhydride. Greater concentrations of dimethylmaleic anhydride are needed to achieve the same extent of acylation produced by methylmaleic anhydride and 57% of the total amino groups in the enzyme preparation are resistant to dimethylmaleylation (Fig. 3). Clear supernatants are obtained by centrifuging the dimethylmaleylated material and they contain less than 10% of the total phospholipid with up to 45% of the total protein but are without Na,K-ATPase activity.

Dimethylmaleic anhydride (mol/equiv. of amino groups)

Fig. 3. Results of treating the Na,K-ATPase preparation with dimethylmaleic anhydride at pH 8.5 in a pH-stat. (a) Protein (■) and lipid phosphorus (□) in the supernatant of centrifuged samples; (b) amino groups (●) and Na,K-ATPase activity (○). Results are expressed as percentages of the unmodified sample that contained 4.4 mg of protein, 4.54 μ mol of lipid phosphorus, 5.4 μ equiv. of amino groups with a Na,K-ATPase activity of 62 μ mol of P_i /h per mg of protein and which was treated similarly.

Concentrations of dimethylmaleic anhydride 20–30-fold in excess of the amino groups in the preparation were not found to have effects greater than those shown in Fig. 3 or to alter the measured content of thiol groups.

Hydrolysis of dimethylmaleyl-amide bonds took place readily under conditions that did not decrease the Na,K-ATPase activity of unmodified material. Exposure of dimethylmaleylated material to pH 7 and 37°C or 4°C for appropriate times caused regeneration of amino groups and partially restored Na,K-ATPase activity (Table 2). There was no significant restoration of Na,K-ATPase activity if more than about 20% of the amino groups were initially modified. The presence of ATP during removal of the dimethylmaleyl groups decreased the residual Na,K-ATPase activity of the acylated material and the additional presence of cations necessary for full enzymic hydrolysis of ATP resulted in complete loss of enzyme activity (Table 3).

Tetrafluorosuccinic anhydride. Reaction of this compound with the amino groups of an insoluble protein at pH 7 gives a soluble derivative from which

Table 2. *Restoration of amino groups and Na,K-ATPase activity to the dimethylmaleylated preparation*

Enzyme preparation equivalent to 4.5 mg of protein was treated with various amounts of dimethylmaleic anhydride at pH 8.5 in a pH-stat. Samples of the acylated preparations were analysed immediately for Na,K-ATPase activity and amino groups or adjusted to pH 7 with KOH and analysed after 72 h at 4°C (Expt. A) or 1 h at 37°C (Expt. B). Results are expressed as percentages of untreated samples which contained 1.1 μ equiv. of amino groups/mg of protein and had Na,K-ATPase activities of 69 μ mol of P_i /h per mg of protein (Expt. A) and 103 μ mol of P_i /h per mg of protein (Expt. B).

Expt.	Dimethylmaleic anhydride (μ mol/equiv. of amino groups)	After acylation		After treatment at pH 7	
		Amino groups (%)	Na,K-ATPase (%)	Amino groups (%)	Na,K-ATPase (%)
A	6.8	91	11	97	30
	11.2	83	2	98	7
	18.8	63	1	99	6
B	5.7	89	10	95	24
	11.9	80	1	93	2
	17.0	63	0	93	0

Table 3. *Effect of ATP and salts on de-acylation of the dimethylmaleylated material*

One sample of dimethylmaleylated material was kept cold at pH 8.5 whereas ATP and salts were added to other samples which were then brought to pH 7 with KOH and incubated at 37°C for 1 h. Final concentrations were: KOH, 2 mM; $MgCl_2$, 2 mM; NaCl, 40 mM; ATP, 2 mM. Results are expressed as a percentage of untreated material which had an Na,K-ATPase activity of 66 μ mol of P_i /h per mg of protein and contained 1.2 μ equiv. of amino groups/mg of protein.

Sample	Additions	pH	Amino groups (%)	Na,K-ATPase activity (%)
1	None	8.5	89	23
2	None	7	96	52
3	ATP	7	93	14
4	$MgCl_2$, ATP	7	94	14
5	NaCl, $MgCl_2$, ATP	7	90	0

the acyl groups are cleaved at pH 9.5 and 0°C (Braunitzer *et al.* 1968). It was found that the reaction of tetrafluorosuccinic anhydride with the Na,K-ATPase preparation could not be quantitated by measuring unchanged amino groups with TNBS, even at pH 7.5, because of the lability of the tetrafluorosuccinyl-amide bonds. A ninhydrin procedure at pH 5 (Rosen, 1957) was also found to be unsatisfactory.

Na,K-ATPase activity of the enzyme preparation was lost after exposure to tetrafluorosuccinic anhydride and the turbidity of the enzyme suspensions was increased (Table 4). Centrifugation of the tetrafluorosuccinylated material gave supernatants containing less protein than was yielded by the unchanged enzyme material, suggesting that tetrafluorosuccinylation caused aggregation of protein constituents. These changes were readily apparent since the tetrafluorosuccinylated enzyme material was visibly flocculent and settled rapidly from suspension. The residual Na,K-ATPase activity

of partially tetrafluorosuccinylated material was not found to be increased when the samples were brought to pH 9.5 and stored at 4°C for 20 h. This treatment was likely to restore amino groups to the acylated material and did not diminish Na,K-ATPase activity of unmodified material. Inability to achieve even partial restoration of Na,K-ATPase activity suggests the enzyme is denatured after reaction with tetrafluorosuccinic anhydride.

1,2,4-Benzenetricarboxylic anhydride. This compound was selected to determine whether introduction of further negative charges would increase the solubility of constituents of the Na,K-ATPase preparation. Additionally it was thought possible that the acyl groups might be removed under mild conditions since benzenetricarboxylic anhydride possesses a stereochemical configuration similar to that of maleic anhydride which facilitates hydrolysis of the maleyl-amide bond (Butler *et al.* 1969).

When present in 50-fold excess over the available amino groups at pH 8.5 benzenetricarboxylic

Table 4. *Treatment of the Na,K-ATPase preparation with tetrafluorosuccinic anhydride*

The enzyme preparation containing 4 mg of protein and equivalent to 5.4 μ equiv. of amino groups was treated with various amounts of tetrafluorosuccinic anhydride at pH 7 and 5°C under pH-stat conditions (see the Experimental section). After dilution to 5 ml the turbidity of the suspensions was measured at 450 nm, Na,K-ATPase activity was determined and 2 ml samples were centrifuged at 150 000g for 1 h for determination of protein and phospholipid in supernatants and deposits.

Tetrafluorosuccinic anhydride (μ mol)	Before centrifugation		After centrifugation			
	Extinction (450 nm)	Na,K-ATPase (μ mol of P_i /h per mg of protein)	Protein (mg)		Phospholipid (μ mol)	
			Supernatant	Deposit	Supernatant	Deposit
0	0.279	103	0.11	1.38	0.44	1.79
50	0.448	14	0.02	1.36	0.37	1.68
110	0.412	0	0.06	1.06	0.37	1.42
160	0.373	2	0.05	1.0	0.45	1.48
205	0.455	2	0.05	1.10	0.43	1.48
252	0.416	0	0.05	1.22	0.39	1.69

Fig. 4. Results of treating the Na,K-ATPase preparation with benzenetricarboxylic anhydride at pH 8.5 in a pH-stat. (a) Protein (■) and lipid phosphorus (□) in the supernatant of centrifuged samples; (b) amino groups (●) and Na,K-ATPase activity (○). Results are expressed as percentages of unmodified material that contained 4.6 mg of protein, 5.2 μ mol of lipid phosphorus, 5.4 μ equiv. of amino groups with an Na,K-ATPase activity of 91 μ mol of P_i /h per mg of protein and which was similarly treated.

anhydride acylates 87% of these groups and there is complete loss of Na,K-ATPase activity (Fig. 4). Supernatants obtained after centrifugation of suspensions of the acylated material contained up

to 35% of the total protein and 15% of the total phospholipid. Exposure of benzenetricarboxylated material to pH 6 or 10 for 3 h at 37°C did not result in the regeneration of amino groups so that there was no possibility of restoring Na,K-ATPase activity to modified material.

DISCUSSION

2,4,6-Trinitrobenzenesulphonic acid appears to react with all possible sites in the Na,K-ATPase preparation and so should provide a reliable indication of the extent of modification of amino groups. The TNBS-reactive substances removed by lipid extraction of the preparation are equivalent to 0.7 μ equiv. of amino groups/mg of protein and are likely to belong principally to phosphatidylserine, phosphatidylethanolamine and ethanolamine plasmalogen. These phospholipids thereby comprise about 65% of the total phospholipid in this sodium iodide-treated Na,K-ATPase preparation (Pull & McIlwain, 1970) whereas they are about 40% of the total in cerebral microsomes (Eichberg, Whittaker & Dawson, 1964). Phosphatidylethanolamine and phosphatidylserine micelles display little reaction with TNBS at pH 7.4–8.9 that is attributed to bonding of amino and phosphate groups (Papahadjopoulos & Weiss, 1969), but this does not appear to be the situation of these phospholipids in this Na,K-ATPase preparation.

The Na,K-ATPase of cerebral microsomes has previously been shown to be inactivated by TNBS (Schwartz, Bachelard & McIlwain, 1962) and inactivation of the sodium iodide-treated Na,K-ATPase has now been shown by acid anhydrides which also modify amino, but not thiol, groups of the preparation. These results are consistent with a

suggestion (Somogyi, 1968) that lysine is at or near the active site for Na,K-ATPase activity but cannot be regarded as evidence for the functional involvement of amino groups in the reaction mechanism since the modifications disrupt the preparation. In this context it is more suggestive that ATP affords partial protection against loss of enzyme activity on methylmaleylation and that both ATP and ouabain alter the rates of trinitrophenylation of the preparation; but such effects could be consequent on the induction of general conformational changes by these compounds. The impurity of the preparation and uncertain molecular weight of the Na,K-ATPase (Kepner & Macey, 1969) preclude establishment of a relationship between loss of activity and introduction of acyl groups into the enzyme. However, Na,K-ATPase activity of the preparation is more sensitive to amino group modification than rabbit muscle aldolase which is 50% inactivated when about 20% of its amino groups are methylmaleylated (Gibbons & Perham, 1970).

The anhydrides examined, other than tetrafluorosuccinic anhydride, caused release of protein with little phospholipid from the preparation in a soluble form, presumably by diminishing ionic protein and protein-lipid interactions and favouring the solubility of constituents by introducing carboxylate groups of greater polarity than the amino groups. Aggregation of the tetrafluoro-succinylated preparation possibly results from the highly polar substituents providing opportunity for formation of polar cross linkages. Dimethylmaleic anhydride was the most effective in causing release of protein although it reacted with fewer amino groups than methylmaleic or benzenetricarboxylic anhydride suggesting that factors other than the introduction of acidic groups are involved. Alkylation or acylation of thiol groups might be expected to further enhance the solubility of protein constituents but there was no detectable loss of thiol groups after reaction of the preparation with methyl- or dimethylmaleic anhydrides. Experimental conditions may not, however, have been optimum for determining release of constituents from the variously modified preparations and it is not known if the same constituents are being released from each derivative of the preparation.

It was disappointing to find that significant amounts of protein are released only when more than about 20% of the amino groups in the preparation were dimethylmaleylated and there is no restoration of Na,K-ATPase activity by procedures hydrolysing the dimethylmaleyl-amide bonds. Similar results have been obtained in restoring enzyme activity to dissociated methylmaleyl aldolase and the irreversible inactivation was associated with loss of thiol groups (Gibbons & Perham, 1970). High concentrations of dimethylmaleic anhydride may

introduce dimethylmaleyl groups into the preparation, other than at amino groups, that are not subsequently removed so preventing full restoration of Na,K-ATPase activity. The Na,K-ATPase may also be inactivated by side reactions peculiar to the dimethylmaleylated preparation that could explain the further loss of Na,K-ATPase activity on removing dimethylmaleyl groups in the presence of ATP and cations. Alternatively, the inability to restore enzyme activity may illustrate the complexity of the Na,K-ATPase system, so that further study will be of value.

I thank Professor H. McIlwain for his help and encouragement, Mr W. S. Chivers for skilled technical assistance and the Research Fund of the Bethlem Royal Hospital and Maudsley Hospital for supporting this work.

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