sisting of a short length of 8 mm. diam. Visking tubing knotted to form a bag, the open end being secured to a piece of glass tubing by a close-fitting rubber stopper fitted to a test tube with a side arm. The bag, after being washed with distilled water, was equilibrated with a drop of the sample solution; this drop was discarded before evacuating the tube and introducing the rest of the sample. Approx. 0.2 ml. of the filtrate was collected and diluted for the determination of the ultrafilterable magnesium. It was found necessary to perform the ultrafiltration with the undiluted sample since dilution affects the distribution between the residue and the filtrate. By equilibrating the membrane with the sample to be filtered the capacity of the membrane to adsorb Mg^{2+} ions is decreased.

RESULTS AND DISCUSSION

The results obtained when the Mg^{2+} ion concentration of the incubation medium was varied between 5 and 13 mM are shown in Fig. 1. Irrespective of the concentration of protein the maximum amount of Mg^{2+} ions bound was reached at about 10 mM, the actual quantity being bound decreasing with the protein concentration. After the optimum concentration of Mg^{2+} ions was reached the percentage of the Mg^{2+} ions in the free state naturally started to rise. As shown by

Campbell *et al.* (1964) the concentration of Mg^{2+} ions at which the stimulation by polyU of phenylalanine incorporation is optimum at 10 mM. It follows that the effect of polyU is optimum when the particulate fraction is saturated with Mg^{2+} ions, excess of Mg^{2+} ions having an inhibitory effect.

When a similar experiment was carried out in which duplicate tubes contained 48 μg . of polyU, the presence of polyU had no significant effect on the percentage of Mg^{2+} ions that was bound by the particles. Under the experimental conditions used 75 % of the polyU was not ultrafilterable. It is concluded that the effect of polyU on the incorporation of phenylalanine is not to be attributed to a change in the amount of Mg^{2+} ions bound to the particles.

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Stimulation and Solubilization of the Sodium Ion-Activated Adenosine Triphosphatase of Cerebral Microsomes by Surface-Active Agents, especially Polyoxyethylene Ethers: Actions of Phospholipases and a Neuraminidase

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(Received 14 November 1963)

Part of the adenosine-triphosphatase activity of cerebral tissues is firmly attached to microsomal material and, by centrifuging cerebral dispersions, can largely be separated from the differently activated adenosine triphosphatases of other subcellular fractions (Deul & McIlwain, 1961; Schwartz, Bachelard & McIlwain, 1962). For many aspects of its further study, however, release from the microsomal structure is necessary and has now been achieved.

Importance is given to the microsomal enzyme through characteristics of its activation by Na^+ and K^+ ions (McIlwain, 1962*a, b*, 1963; Aldridge, 1962), which connect it and similarly activated

enzymes in other tissues (Skou, 1957; Dunham & Glynn, 1961; Bonting, Simon & Hawkins, 1961; Wheeler & Whittam, 1962) with a possible role in active cation movement. Some reflexion of this function might be found in relationships between the enzyme and microsomal structures, and be displayed on its release; thus a suggestion of how the enzyme performs a role in ion movement postulates particular relationships between the enzyme and membrane structures (McIlwain, 1962*a*, 1963). It was, therefore, especially interesting to find that certain surface-active agents, before they released the adenosine triphosphatase, enhanced its activity, and did so specifically in the presence of sodium

salts (Swanson & McIlwain, 1963). This phenomenon has been further studied with a series of structurally related polyoxyethylene ethers.

The relationship between the adenosine-triphosphatase activity and membrane components has been explored also by treating the microsomal fraction with hydrolytic enzymes. A neuraminidase was used because the fraction carried neuraminic acid-containing proteins and lipids; the latter, the gangliosides, are enriched in the fraction and may be related to cation movement (Wherrett & McIlwain, 1962). Phospholipases A and C were used because lecithins and sphingomyelins are major components of the microsomal fraction.

EXPERIMENTAL

Preparation and treatment of microsomal fractions

Preparation. The grey matter of guinea-pig cerebral hemispheres was dispersed and fractionated by differential centrifugation, as described by Schwartz *et al.* (1962). Microsomal suspensions so obtained usually contained 2.0–2.5 mg. of protein/ml., representing about 12–15 mg. from 1 g. of grey matter. The microsomal suspensions were stored at -20° in 0.32M-sucrose, and retained adenosine-triphosphatase activity for several months. Fractions were examined as a routine for protein content, which closely paralleled the values of the previous authors, and occasionally microscopic examination was carried out after staining with Janus green. This indicated the absence of mitochondria. Mr A. Trevor, using the method described by Schwartz *et al.* (1962), found succinate-dehydrogenase activities of 6.5 and 9.8 μ moles/g. of cortex/hr. in representative microsomal fractions, corresponding to less than 2% of that in the original homogenate. Microsomal RNA, determined by the method of Littlefield, Keller, Gross & Zamecnik (1955), ranged from 215 to 480 μ g./g. of cortex (9–15% of that in the original tissue).

Treatment with detergents. Microsomes from about 1 g. of guinea-pig cortex (about 15 mg. of protein) were exposed in an ice bath for approx. 15 min. to a solution of detergent which, unless otherwise specified, was in 0.32M-sucrose and at pH 7.4. After centrifugation in the no. 40 head of the Spinco model L preparative ultracentrifuge at 105000g for 60 min., the supernatant was removed and the pellet resuspended in a homogenizer in 0.32M-sucrose. Enzyme assays were performed within 2 hr. of treatment.

Treatment with neuraminidase. The enzyme was obtained from Behringwerke A. G., Marburg-Lahn, W., Germany, and was a partially purified preparation isolated from culture filtrates of *Vibrio cholerae*. Proteolytic and aldolase activities had been removed and 1 ml. of the preparation was stated to be capable of releasing 100 μ g. of *N*-acetylneuraminic acid in 15 min. at 37° (100 units). Before use, the enzyme preparation was dialysed against water in the cold room. The microsomal fraction was exposed to the neuraminidase as described in Table 4, after which portions of the exposed mixtures were pipetted into 1 ml. of 0.1M-sodium iodoacetate at pH 7 (this reagent was used because the trichloroacetic acid normally employed caused loss of microsomal *N*-acetylneuraminic acid). After centrifugation at 35000g for 20 min., the clear supernatant was

removed by decantation and the pellets were drained. Pellets were extracted with 10 ml. of chloroform-methanol (2:1, v/v) and kept for 2 hr. at room temperature before filtration into fresh glass-stoppered tubes through sintered glass. Then 2 ml. of 0.1M-KCl was added to the mixture, which was shaken and centrifuged at 5000g for 5 min. The lower phase was shaken with 0.2 vol. of 'upper phase mixture' (0.1M-KCl-methanol-chloroform; 47:48:3, by vol.). After centrifuging as above, the upper phases were combined and dialysed against three changes of 30 l. of distilled water for 4 days at 4° to remove all remaining sucrose. The dialysed volumes were evaporated to dryness under a stream of air at 60–80 $^{\circ}$, 1 ml. of 0.1N-H₂SO₄ was added and the solution was incubated at 80 $^{\circ}$ for 2 hr. Samples (0.5 ml.) were then removed for the thiobarbituric acid assay of *N*-acetylneuraminic acid (Aminoff, 1961). The recovery of *N*-acetylneuraminic acid was calculated from the extinction at 549 m μ , corrected for the extinction at 532 m μ , according to Warren's (1959) method.

Treatment with phospholipases. Russell-viper venom was the source of phospholipase A. Phospholipase C, from L. Light and Co., Colnbrook, Bucks., was prepared from culture filtrates of *Clostridium welchii* by ammonium sulphate precipitation and freeze-drying (Macfarlane & Knight, 1941). The activity of the filtrates as phospholipases was examined by thin-layer chromatography: 1 ml. samples from the enzyme reaction mixtures were added to 1 ml. of 5% (w/v) trichloroacetic acid and, after approx. 30 min. at 0 $^{\circ}$, were centrifuged at 5000g for 15 min.; the supernatants were removed and the pellets drained. Lipid extracts were prepared from the pellets and chromatographed on thin layers of silicic acid, as described by Bradford, Swanson & Gammack (1964). Trichloroacetic acid-soluble phosphate was also measured after treatment of microsomes by phospholipase C: 0.5 ml. of the trichloroacetic acid supernatant was evaporated to dryness at 60–80 $^{\circ}$ under a stream of air, and digested with boiling 60% (w/v) perchloric acid until clear (15–20 min.). Phosphate was then measured according to the method of Martin & Doty (1949).

Antisera to *C. welchii* were examined for their effect on inhibition caused by phospholipase C. An amount of the antiserum K3663 (Table 5) was chosen that was sufficient to prevent formation of turbidity when a given amount of phospholipase C was incubated for 30 min. at 37° with an emulsion of egg lecithin (Merck) in the medium of Macfarlane & Knight (1941). It was found that 0.75 unit neutralized 0.1 mg. of phospholipase C, and other antisera with known content of α -antitoxin were then diluted to provide comparable titres.

Assays and analyses

Assay of adenosine triphosphatase. The standard reaction mixture of 1 ml. contained (final concentrations): tris-HCl buffer, pH 7.4 (30 mM), KCl (30 mM), MgCl₂ (3 mM), ATP (tris salt) (3 mM) and NaCl (100 mM). When NaCl was omitted, the concentration of the tris-HCl buffer was increased to 130 mM to maintain approximate isosmolality. After preincubation of the mixture with shaking at 37° for 5 min., the reaction was begun by adding the microsomes to be assayed with a micropipette, usually in a volume of 0.05 ml. containing 0.025–0.075 mg. of microsomal protein. The reaction was stopped after 5 min. by adding 1.2 ml. of 5% (w/v) trichloroacetic acid. Determinations were done in duplicate. In some instances (noted) the reaction was

begun by adding ATP, or by using a single tube and withdrawing samples at intervals for phosphate determination. The tubes were kept on ice, usually for 1–2 hr., before the phosphate determinations.

Assay of cholinesterase. The manometric method of Aldridge & Johnson (1959) was used. The medium contained (final concentrations): NaCl (120 mM), MgCl₂ (32 mM), NaHCO₃ (28.6 mM), acetylcholine (13.8 mM), sucrose (69 mM) and microsomes (0.84 mg. of protein/flask) in 3.5 ml., equilibrated with CO₂ + N₂ (5:95).

Determination of protein. The determinations were performed according to the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin (Armour and Co., Eastbourne, Sussex) as standard. In the presence of polyoxyethylene detergents, a fine precipitate appeared on the addition of the Folin-Ciocalteu reagent. Tubes were therefore centrifuged for 5 min. at 1500g before measurement of the extinction. The presence of detergent was found not to alter the extinction of standard protein solutions when the precipitate was deposited in this fashion.

Determination of phospholipid phosphorus. This was determined as described by Bradford *et al.* (1964). Saturated aqueous KCl (0.5 ml.) was added to the 1.3N-perchloric acid used as precipitant when determinations were carried out in the presence of high concentrations of polyoxyethylene detergents.

Determination of inorganic phosphate. The Martin & Doty (1949) and Lindberg & Ernster (1956) adaptation of the Berenblum & Chain (1938) method was followed, with minor modifications. To a glass-stoppered test tube containing 0.5 ml. of 5N-H₂SO₄, 0.5 ml. of 10% (w/v) ammonium molybdate and 3 ml. of isobutanol-benzene (1:1, v/v) mixture, was added a 2 ml. portion of the sample. When smaller portions were used, water or 5% (w/v) trichloroacetic acid was added to bring the total volume of the aqueous phase to 3 ml. The concentration of acid in the mixture was found to be of importance in determining the amount of phosphomolybdate extracted, as mentioned by Berenblum & Chain (1938). If the final acid concentration exceeded 2.5N, less than 25% of the phosphate was extracted into the organic phase. The tubes were shaken for 30 sec. and usually briefly centrifuged to facilitate separation of the two phases. A portion, usually 2 ml., was removed with a syringe pipette, and 3.2% (v/v) H₂SO₄ in ethanol was added to make a final volume of 5 ml. The phosphomolybdate was reduced with 0.5 ml. of a freshly prepared dilution of 10% (w/v) SnCl₂ in conc. HCl with 200 vol. 0.5N-H₂SO₄. After immediate mixing, the blue colour was allowed to develop for 20 min. and its intensity was measured in a Unicam model SP. 600 spectrophotometer at 650 mμ.

Light-scattering and ultrafiltration studies. Light-scattering of microsomal suspensions at concentrations of approx. 0.02 mg. of protein/ml. was measured through 90° in an Aminco-Bowman spectrophotofluorometer with the incident beam at 350 mμ. Ultrafiltration studies were carried out with suspensions and detergent-treated extracts at concentrations of 0.7–2.3 mg. of protein/ml. These were placed above samples of a series of graded filters (Membranfilter, Göttingen, Germany), supported on sintered-glass filters and under suction at 4°. Protein and adenosine-triphosphatase activity were determined in the original suspensions and extracts, and in the collected filtrates.

Binding of sodium and potassium ions by microsomes. Microsomal suspensions were centrifuged for 60 min. in the SW39 head of the Spinco model L centrifuge at 100000g. The supernatant was decanted, and the tube was drained and wiped with filter paper. The deposit was dispersed in sucrose in a glass homogenizer tube before assay. Sucrose used for microsomal suspensions were about 60 μM with respect to tris, added in taking the sucrose to pH 7.4. After exposure to 6% (w/v) trichloroacetic acid for 15 min. the suspension was centrifuged for 10 min. at 2000g, the supernatant decanted, and determination of Na⁺ and K⁺ ions performed on an EEL model A flame photometer.

Reagents

Reagents not otherwise specified were of highest commercial grade. Solutions of ATP (tris salt) were prepared according to the method of Schwartz *et al.* (1962) and stored at a concentration of 30 mM in a deep-freeze. Solutions 3 mM with respect to ATP were found to contain 0.10–0.14 m-equiv. of Na⁺ ion/l. and 0.02–0.03 m-mole of inorganic phosphate/l. Lubrol W (Imperial Chemical Industries Ltd., 1959) and cetyltrimethylammonium bromide (Cetavlon) were from Imperial Chemical Industries Ltd., Wilmslow, Cheshire. Sodium deoxycholate was from L. Light and Co. Ltd. Tris 121 (Sigma Chemical Co., St Louis, Mo., U.S.A.) was used in all enzyme assays. Digitonin was AnalaR grade from British Drug Houses Ltd., and varied in solubility from batch to batch. The calcium salt of phosphorylcholine chloride was a gift from L. Light and Co. Ltd., Colnbrook, Bucks.

Several members of a series of polyoxyethylene ethers of the general structure R·[O·CH₂·CH₂]_n·OH were kindly given by Dr A. Spinks of Imperial Chemical Industries Ltd. (Pharmaceuticals Division), Alderley Park, Cheshire. These are described below (e.g. in Fig. 5) in terms of the average number of ethylene glycol residues, *n*. The members from *n* average 16 to *n* average 29 were ethers of cetyl alcohol; those from *n* average 2.5 to *n* average 13 were mixtures of ethers of cetyl alcohol and oleyl alcohol. The batch of Lubrol W used was of this general structure and with *n* average 16; but it is emphasized by the suppliers that the name is of a proprietary product rather than a definite substance. Cetomacrogol 1000 was of this general structure and with *n* average 24; it was from Glovers Chemicals Ltd., Wortley, Leeds, 12, being given by Dr P. H. Elworthy (see Elworthy & Macfarlane, 1963).

Diagnostic antisera to *C. welchii* were generously supplied by Dr I. Batty, of Wellcome Research Laboratories, Beckenham, Kent, who also suggested the type of experiment reported in Table 5. These antisera were chosen to differ in amounts of titrated antibodies: antiserum K3663 (Type A) contained 300 units/ml., K3674 (type C) contained 12 units/ml. and K3736 (type E) contained 13 units/ml.

RESULTS

Adenosine-triphosphatase assay and microsomal stability

The course of liberation of inorganic phosphate from ATP by cerebral microsomal fractions is shown in Fig. 1. In these reaction mixtures, which contained ATP (magnesium salt) with sodium,

potassium and tris salts but no non-electrolytes, the characteristic activation of the system by Na^+ ions is well exhibited. However, the rate of reaction fell considerably during the 30 min. of measurement, and the basis for the fall was examined. The reaction rate decreased both with and without sodium chloride, although in the latter instance the fall occurred somewhat more rapidly, so that the degree of stimulation by Na^+ ions appeared greater with longer incubation times. If sodium chloride was added to the reaction mixture after the reaction had run for 15 min., the rate increased to that of microsomes which had been incubated from the beginning in the presence of sodium chloride (Fig. 1). Thus the difference in degree of inactivation did not appear to reflect a protective effect of Na^+ ions. Since less than 5% of the total ATP was usually hydrolysed, the decrease in velocity seemed unlikely to be caused by a lowering of the effective substrate concentration. Further, when microsomes were present in greater concentrations (1 mg. of protein/ml. of reaction mixture), so that 40% of the ATP was split in 60 sec., the velocity approached linearity for this period of time and corresponded with the same rate per unit weight of protein. Inhibition by reaction products also seemed unlikely, as the velocity was not altered by the addition of further inorganic phosphate (0.5 mM) or ADP (1.3 mM), concentrations greater than those normally produced during the reaction. The addition of egg albumin or serum albumin (0.1%), glutathione

Fig. 1. Time-course of liberation of inorganic phosphate from ATP by microsomal suspensions at 37°C . The reaction mixtures contained ATP (tris salt) (3 mM), MgCl_2 (3 mM), KCl (30 mM) and tris-HCl buffer, pH 7.4 (30 mM), with microsomal protein (60 μg). A, With NaCl (100 mM). B, Without NaCl; NaCl was added to give 100 mM after 15 min. incubation (arrow); the extension of curve B is based on replicate tubes run with no added NaCl.

(3 mM), L-cysteine (3 mM) or EDTA (1 mM) failed to alter the time-course.

On the other hand, when microsomes were preincubated in solutions containing electrolytes (Fig. 2b), and samples removed from the solutions at intervals and their adenosine-triphosphatase activities assayed, this activity fell in a fashion that did not occur if the preincubation had been in 0.32M-sucrose. It was noted also that microsomes suspended in the electrolyte solutions aggregated and fell out of suspension. This change in physical characteristics of microsomal suspensions was

Fig. 2. (a) Microsomal preparations containing 0.02 mg. of protein were suspended at room temperature in 1 ml. of medium containing: ●, sucrose (0.32M); ○, tris-HCl buffer, pH 7.5 (150 mM), or NaCl (160 mM), or a solution containing MgCl_2 (3 mM), KCl (30 mM), NaCl (100 mM) and tris-HCl buffer, pH 7.4 (30 mM), with or without ATP (3 mM); the 90° light-scattering was then measured at $360\text{ m}\mu$ at the intervals indicated. (b) Effect of preincubation of a microsomal suspension on its adenosine-triphosphatase activity; the suspension contained 1.73 mg. of protein in 2 ml. of medium containing: ●, sucrose, pH 7.5 (0.32M); ○, tris-HCl buffer, pH 7.5 (150 mM), or a solution containing MgCl_2 (3 mM), KCl (30 mM), NaCl (100 mM) and tris-HCl buffer, pH 7.5 (30 mM); after incubation at 37° for the indicated periods, 0.05 ml. samples were withdrawn and assayed for adenosine-triphosphatase activity in the presence of NaCl (100 mM).

further examined by following the course of light-scattering through 90° in a spectrophotofluorometer. A decrease in scattering was seen, roughly paralleling in its time-course the inactivation of the adenosine triphosphatase. The change in light-scattering, moreover, was absent when the particles were suspended in sucrose or in water, and present when they were suspended in the reaction mixture or in sodium chloride, potassium chloride, or tris buffer (Fig. 2). The effect did not resemble the ATP-dependent contraction of swollen mitochondria (Lehninger, 1962), since it was not influenced by the addition of ATP (3 mM). It led to the use of a 5 min. reaction time in determining adenosine-triphosphatase activity in subsequent experiments, though conditions were later found (Fig. 6) in which the adenosine triphosphatase of the suspensions was stable in salt solutions.

Actions of surface-active agents

Detergents were previously examined (Schwartz *et al.* 1962) as a means of solubilizing the adenosine triphosphatase, and some success was achieved, though the different conditions then employed gave results that differ in detail from those now reported (Table 1). Both studies employed initially the same criteria of solubilization, namely lack of deposition on centrifuging at 110 000g for 60 min. The microsomal fraction used as starting material was deposited at 20 000g in 60 min. Agents that solubilized caused also a visually evident clearing of the microsomal suspension.

Two ionic detergents. The cationic detergent cetyltrimethylammonium bromide, at a low concentration (0.01%), solubilized a negligible proportion only of the microsomal protein or enzyme activity. The detergent had, however, an action on that part of the enzyme that remained insoluble: its adenosine-triphosphatase activity in the absence of Na⁺ ions was increased, although this activity was now accelerated only about 10% by sodium chloride, in contrast with the acceleration of 50% before treatment (Table 1). With increase in detergent concentration, more protein and enzyme activity were found in the supernatant, but a considerable net loss or inhibition of enzyme activity occurred. In neither of these cases was the solubilized adenosine triphosphatase stimulated by sodium chloride. A microsomal adenosine triphosphatase was observed by Schwartz *et al.* (1962) to be stimulated by basic polypeptides, and also cationic reagents, in the absence of Na⁺ ions and while still not solubilized. The present action of cetyltrimethylammonium bromide thus appears likely to be independent of its detergent properties.

Sodium deoxycholate was examined as an example of an anionic surface-active agent. At 0.05% (Table 1) and also at 0.008% (not quoted),

Table 1. *Effects of detergents on microsomal adenosine triphosphatase, protein and phospholipid*

Detergent	Concn. of detergent (% w/v)	Protein (mg./tube)		Phospholipid P (μg./tube)		Adenosine-triphosphatase activity (μmoles of inorganic phosphate liberated/hr./tube)					
						Original microsomes, no detergent		Supernatant, after detergent		Deposit, after detergent	
		Super-natant	Deposit	Super-natant	Deposit	Without NaCl	With NaCl (100 mM)	Without NaCl	With NaCl (100 mM)	Without NaCl	With NaCl (100 mM)
Cetyltrimethylammonium bromide	0.01	<0.5	9.7	<5	314	353	522	<5	<5	399	436
	0.1	6.8	2.1	165	115	353	522	18	26	49	44
Sodium deoxycholate	0.05	1.1	12.6	3	310	345	655	21	17	260	499
	0.2	7.6	5.8	113	240	345	655	31	88	200	625
Digitonin	0.2	3.0	7.8	—	—	303	438	—	140	372	980
	1.0	5.3	5.1	—	—	303	438	—	312	259	405
Polyoxyethylene ether (n av. 16)	0.1	3.1	7.0	178	400	256	447	55	195	187	497
Polyoxyethylene ether (n av. 10)	0.2	8.2	6.0	—	—	244	555	122	723	55	104

Microsomal fractions from about 1 g. of guinea-pig cortex (their quantity differing in the different experiments, as indicated by the protein contents quoted) were treated in 12 ml. of 0.32M-sucrose for 15 min. at 0°, centrifuged at 105 000g for 60 min., and determinations made on supernatant and resuspended deposit. Values in this Table are selected from a range of three to seven concentrations of each detergent. Fuller information on treatment with cetyltrimethylammonium bromide, deoxycholate and digitonin is reported by Bradford *et al.* (1964). The polyoxyethylene ethers are characterized by the average number, *n*, of polyoxyethylene residues/molecule, and further details of their effects are given in the text.

the protein and enzyme activity solubilized were small, but the total activity in supernatant and deposit was markedly less than in the original suspension. At 0.2% (4.8 mM), deoxycholate solubilized less than 15% of the enzyme activity, although 57% of the microsomal protein was recovered in the supernatant. No significant activation of the enzyme was seen, but its activity in the absence of Na^+ ions was diminished, and a greater proportional stimulation by added sodium chloride thus resulted (cf. Skou, 1962).

Non-ionic detergents. Digitonin and polyoxyethylene ethers (see the Experimental section) were more promising as solubilizing agents for the microsomal adenosine triphosphatase. After exposure and centrifuging, stimulation by sodium chloride was retained in both supernatant and deposit (Table 1). In further contrast with the ionic agents, digitonin and the polyoxyethylene

ethers both increased the adenosine-triphosphatase activity in presence of sodium chloride. In the absence of sodium chloride, the polyoxyethylene ethers were either without effect or they inhibited the adenosine triphosphatase, whereas digitonin was found, from the experiments of Table 1 and others, to stimulate it. The effects of a range of concentrations of Lubrol W, one of the polyoxyethylene ethers, are shown in Fig. 3. Between 1 and 4 mg. of detergent/ml., increasing proportions of each constituent measured—phospholipid, protein and adenosine triphosphatase—remained in the supernatant on centrifuging; the proportions of each so solubilized were similar, reaching 45–55% at 4 mg. of Lubrol W/ml., and remaining at 40–60% at 8 mg. of Lubrol W/ml.

Marked differentiation between properties of Lubrol W in activating and in solubilizing the adenosine triphosphatase are shown in Fig. 3, for activation was maximal at 1 mg. of Lubrol W/ml., when only 28% of the enzyme was not deposited by centrifuging. At 4 mg. of Lubrol W/ml., when solubilization of the enzyme was maximal, the detergent was inhibiting the enzyme and not activating it. The amount of Lubrol W necessary for a given action on the adenosine triphosphatase depended on the quantity of microsomal material employed. Thus, in the experiment of Fig. 4, maximal activation of the enzyme was given by 20 μg . of Lubrol W/ml., but in both Figs. 3 and 4 the weight of Lubrol W needed for the activation was approximately equal to the weight of microsomal protein or lipid present in the reaction

Fig. 3. Effect of the concentration of Lubrol W (polyoxyethylene ether of n average 16) on (a) adenosine-triphosphatase activity in the presence (A) and absence (B) of NaCl (100 mM) and (b) solubilizing of cerebral microsomal constituents, namely protein (■), phospholipid (●) and adenosine triphosphatase (○). A microsomal suspension containing 10.4 mg. of protein (0.9 mg./ml.) and 615 μg . of phospholipid phosphorus was exposed to detergent for 15 min. at 0° before centrifugation at 105000g for 60 min. Adenosine triphosphatase activities represent the sum of activities in supernatant and deposit.

Fig. 4. Effect of Lubrol W (n average 16) on adenosine-triphosphatase activity in the presence (A) and absence (B) of NaCl (100 mM). Microsomal fractions containing 26 μg . of protein were exposed for 15 min. at 0° in reaction mixtures containing the concentrations of Lubrol W indicated. After a further 5 min. at 37°, the adenosine-triphosphatase reaction was begun by the addition of ATP (tris salt); the final volume was 1 ml.

mixture. Differentiation between solubilization and stimulation was brought out also by exposing increasing microsomal concentrations to a fixed quantity of polyoxyethylene ether. In one experiment, trebling the quantity of microsomes lowered the proportion of adenosine triphosphatase solubilized from 52 to 15%, whereas the activity of the Na^+ ion-activated enzyme increased from 125 to 240% of its original value.

Polyoxyethylene ethers. Several homologues of Lubrol W of the general formula

were examined (here R is cetyl or a mixture of oleyl and cetyl radicals, and n is the average number of glycol residues; see the Experimental section). Their actions in stimulating the adenosine triphosphatase and in solubilizing the microsomal protein and lipid are shown in Fig. 5; their action

in each respect depended on the length of the polyoxyethylene chain. Compounds with an n average of 6 or less had little action at the concentrations examined, which were approximately equimolar. Those of n average 8 or 10 solubilized most effectively the protein, cholesterol, phospholipid and adenosine triphosphatase of the suspensions. These members were, however, only moderately effective in activating the adenosine triphosphatase; here the member of n average 13 was most active. At 0.2%, the member of n average 10 solubilized 70–90% of adenosine triphosphatase of microsomes derived from 1 g. of cortex (Table 1).

The polyoxyethylene ethers affected not only the ultracentrifugal deposition of microsomal material but also other properties dependent on particle size. After treatment of the suspensions with the polyoxyethylene ether of n average 10 at a concentration of 2 mg./ml., ultrafiltration showed that enzyme activity and protein passed through selectively permeable membranes with pore sizes of 35–100 $\text{m}\mu$. In contrast, no activity and negligible protein was recovered in the filtrates obtained from the passage of original microsomes through membranes with pore sizes up to 500 $\text{m}\mu$. Also, the addition to a microsomal suspension of an amount of detergent sufficient to render 50–60% of its adenosine-triphosphatase activity non-sedimentable decreased its light-scattering. The decrease was most rapid in the first 2–3 min., and continued to take place more gradually during the subsequent 15 min. Clearing, evident visually, followed a similar time-course.

Treatment of microsomal suspensions with the polyoxyethylene ethers altered the time-course of the adenosine-triphosphatase reaction, as well as increasing its velocity. In contrast with the fall-off in reaction velocity shown by untreated microsomes, detergent-treated material hydrolysed ATP at uniform speed for 20–30 min. (Fig. 6). Further, the addition of detergent to microsomal suspensions whose activity had decreased by 75% restored the reaction velocity to values equal to and in some instances greater than the original initial velocity (Fig. 6).

Relationship to sodium ions. As the presence or absence of sodium chloride has conditioned the behaviour of most of the detergents towards the microsomal adenosine triphosphatase, possible interactions with Na^+ ions were investigated in three further ways.

(a) The Na^+ ion-dependence of the reaction was examined in more detail in the presence and absence of Lubrol W. With 0.65 mg. of Lubrol W/ml., the concentration of sodium chloride needed for half maximal activity was about 12.5 mM with or without the detergent (Fig. 7).

Fig. 5. Extraction of microsomes with Lubrol homologues of general formula $\text{R} \cdot (\text{O} \cdot \text{CH}_2 \cdot \text{CH}_2)_n \cdot \text{OH}$, where R was cetyl for compounds with n average 16–29, and a mixture of oleyl and cetyl for n average 2.5–13 (see the Experimental section). The detergent concentration, adjusted according to estimated molecular weight, was 1.3 mM, and microsomal suspensions contained a mean of 13.4 mg. (s.d. $\pm$ 0.64 mg.) of protein/tube, and 319 μg . (s.d. $\pm$ 20 μg .) of phospholipid phosphorus/tube. The adenosine triphosphatase of the untreated microsomes liberated 46 ± 8 μmoles of inorganic phosphate/mg. of protein/hr. in the presence of NaCl (100 mM) and 31 ± 2 $\mu\text{moles/mg.}/\text{hr}$. in the absence of NaCl. The total cholesterol, measured in one experiment, was 2.6 mg. (s.d. $\pm$ 0.2 mg.). The numbers of experiments are given in parentheses in (b). The vertical bars represent s.d. for four experiments. (a) Percentage solubilization of protein; (b) percentage solubilization of adenosine triphosphatase; (c) percentage solubilization of cholesterol (●) and phospholipid (○); (d) adenosine-triphosphatase activities in the presence (A) and absence (B) of NaCl (100 mM).

(b) The increased adenosine-triphosphatase activities shown by cerebral microsomal fractions in the presence of sodium chloride are selectively inhibited by the glycoside ouabain (Schwartz *et al.* 1962; Aldridge, 1962), a phenomenon that is paralleled in other systems and that has been employed in assay of the Na^+ ion-dependent adenosine triphosphatases (Bonting *et al.* 1961). This inhibition with the present microsomal preparations is shown in the

first row of Table 2; the second row shows that the same phenomenon is reproduced in the presence of Lubrol W, though in this case the effects of both sodium chloride and ouabain were greater. Thus the increase with sodium chloride was 41 % without and 120 % with Lubrol W; the inhibition by $10\text{ }\mu\text{M}$ -ouabain was 21 % without and 44 % with Lubrol W.

(c) Direct measurements were made of the Na^+ and K^+ ions associated with the present microsomal suspensions before and after they had been treated with polyoxyethylene detergents (Table 3). This showed that the detergents diminished both the Na^+ and the K^+ ions that were associated with the microsomal pellets. Lubrol W and Cetomacrogol 1000 showed this behaviour. In associated experiments, the concentrations of the Na^+ and K^+ ions of the system were altered by making small additions of sodium chloride or potassium chloride to the microsomal suspensions (Table 3). These additions increased the amount of corresponding ion held by the pellet; the Na^+ ion: K^+ ion ratio in the pellet was similar to the ratio in the supernatant.

Acetylcholinesterase. The effect of Lubrol W on another particle-bound enzyme, acetylcholinesterase, was also examined. In contrast with the stimulation of Na^+ ion-activated adenosine triphosphatase, no consistent change was found, the rates averaging $8.7\text{ }\mu\text{moles/mg. of protein/hr.}$

Fig. 6. Course of liberation of inorganic phosphate from ATP in the presence of polyoxyethylene ether (n average 10). (a) The reaction mixture contained NaCl (100 mM), KCl (30 mM), tris-HCl buffer, pH 7.4 (30 mM), and ATP (tris salt) (3 mM), in a final volume of 20 ml. The reaction was begun by adding microsomes containing 1.2 mg. of protein, and 1 ml. samples were withdrawn at indicated times and added to 5% trichloroacetic acid. After 30 min., 0.6 mg. of detergent in 0.1 ml. of sucrose was added (arrow). (b) Time-course with detergent extract, containing 6 mg. of polyoxyethylene ether and 1.1 mg. of protein in a final volume of 10 ml. Microsomes had been exposed to the detergent for 15 min. at 0° and centrifuged for 1 hr. at 105000g before assay of the supernatant. The liberation of inorganic phosphate was measured in the presence (A) and absence (B) of NaCl (100 mM). The reaction conditions were otherwise similar to those in (a).

Fig. 7. Activation of adenosine triphosphatase by Na^+ ions in the presence (●) and absence (○) of Lubrol W. Adenosine-triphosphatase activity is plotted against the NaCl concentration. Samples (0.05 ml.) of microsomal suspensions containing 0.56 mg. of protein/ml. in the presence and absence of 0.65 mg. of Lubrol W/ml. were assayed in a reaction mixture containing tris-HCl buffer, pH 7.4 (30 mM), ATP (tris salt) (3 mM), KCl (30 mM) and MgCl_2 (3 mM), with the indicated concentrations of NaCl, in a final volume of 1 ml. Lubrol increased the maximal velocity from 56 to 77 μmoles of inorganic phosphate liberated/mg. of protein/hr. The amount of NaCl required for half-maximal activation was 12.5 mM in the presence and absence of detergent.

Table 2. *Effect of ouabain on adenosine-triphosphatase activation by Lubrol W*

Microsomes (0.56 mg. of protein) were incubated with 0.65 mg. of Lubrol W in 2.5 ml. for 15 min. at 0°. Portions (0.05 ml.) were transferred to reaction mixtures with and without ouabain for adenosine-triphosphatase assay.

Adenosine-triphosphatase activity
(μ moles of inorganic phosphate liberated/mg. of protein/hr.)

Added detergent	Without NaCl	With NaCl (100 mM)	With NaCl (100 mM) and ouabain (10 μ M)	With NaCl (100 mM) and ouabain (100 μ M)
None	41	58	46	45
Lubrol W	35	77	43	34

Table 3. *Binding of sodium and potassium ions by microsomes*

Microsomal suspensions containing 4.0–6.8 mg. of protein in 5 ml. with the additions noted were kept for 15 min. at 0°, and then centrifuged in the SW 39 rotor of the Spinco model L centrifuge at 100000g for 60 min. The supernatants were decanted, the tubes drained and wiped with filter paper, and the pellets resuspended in sucrose. The sucrose used contained approx. 0.06 μ mole of tris/ml., except for Expt. 3, where sucrose had been brought to pH 7.4 with KOH. The wet weight of the microsomal pellet was 15.2 ± 2.4 mg./mg. of protein (eight determinations).

Composition after exposure and centrifuging

Expt. no.	Added substance	Composition after exposure and centrifuging			
		Pellet		Supernatant	
		Na ⁺ ions (μ equiv./mg. of protein)	K ⁺ ions (μ equiv./mg. of protein)	Na ⁺ ions (μ equiv./tube)	K ⁺ ions (μ equiv./tube)
1	None	0.083	0.135	1.36	2.38
	KCl (1.5 μ moles)	0.073	0.159	1.43	3.04
	NaCl (0.8 μ mole)	0.106	0.139	2.21	2.54
2	None	0.068	0.117	1.54	2.36
	NaCl (5 μ moles)	0.113	0.103	6.13	2.42
	Lubrol W (0.05 %)	0.046	0.060	1.59	2.40
3	None	0.083	0.191	0.89	1.89
	NaCl (1.6 μ moles)	0.113	0.172	2.53	1.86
	Lubrol W (0.025 %)	0.053	0.133	0.86	1.86
4	None	0.073	0.127	1.41	2.53
	Cetamacrogal 1000 (0.1 %)	0.051	0.054	1.38	2.39
	Cetamacrogal 1000 (0.1 %)	0.042	0.069	1.37	2.46

without addition or with Lubrol W concentrations ranging from 0.014 to 0.14 %, and with 0.84 mg. of microsomal protein in 3.5 ml.

Lipid-splitting enzymes on the microsomal adenosine triphosphatase

Three enzymes were examined for their effects on the Na⁺ ion-stimulated adenosine triphosphatase (Table 4). Neither neuraminidase, which splits *N*-acetylneuraminic acid from microsomal constituents, nor phospholipase A, which removes the β -fatty acid from lecithin, significantly altered the adenosine-triphosphatase activity. Activation with Na⁺ ions was retained after treatment. In contrast, phospholipase C, which removes phosphorylcholine from lecithin and also acts on sphingomyelin (Hanahan & Vercamer, 1954), caused 40 % inhibition in 30 min. Activity both with and without Na⁺ ions was lowered.

The lipolytic action of the enzymes during these experiments was shown by analysis and by thin-

layer chromatography. After treatment with phospholipase A, a fast-moving band, presumably free fatty acid, appeared chromatographically, and the lecithin and phosphatidylethanolamine bands were markedly decreased. After treatment with phospholipase C, a decrease in the lecithin band was again seen and, further, acid-soluble phosphorus rose from 88 μ g./g. of original tissue to 137 μ g./g. after 30 min. and to 252 μ g./g. after 60 min. As lecithin accounts for about 37 % of the phospholipid phosphorus of microsomes (H. F. Bradford, unpublished work), 118 μ g. of lecithin phosphorus would be present in the 320 μ g. of phospholipid phosphorus that the suspension contained. The additional phosphate liberated by phospholipase C probably came from sphingomyelin, though no demonstrable decrease was seen on thin-layer chromatography. The *N*-acetylneuraminic acid of microsomes was diminished from 13.6 to 6.7 μ g./mg. of protein after 60 min. treatment with neuraminidase, indicating that this enzyme too, was

Table 4. *Exposure of microsomal adenosine triphosphatase to neuraminidase, phospholipase A or phospholipase C*

The mixtures used for exposure to the enzymes were as follows. Expt. 1: neuraminidase (dialysed against water overnight at 3°) (500 units), CaCl_2 (2.2 mM), tris-HCl buffer, pH 7.4 (40 mM), and microsomal protein (25 mg.); Expt. 2: neuraminidase (250 units), CaCl_2 (0.9 mM), tris-HCl buffer, pH 7.4 (100 mM), and microsomal protein (9 mg.); Expt. 3: Russell-viper venom (1.5 mg.), either heated at 100° for 15 min. or unheated, CaCl_2 (5 mM), NaCl (250 mM), and microsomal protein (12 mg.); Expt. 4: phospholipase C (100 $\mu\text{g.}$) with microsomal protein (2.4 mg.), in CaCl_2 (100 mM), tris-HCl buffer, pH 7.4 (120 mM); Expt. 5: phospholipase C (either 5 $\mu\text{g.}$ or 100 $\mu\text{g.}$) with CaCl_2 (20 mM), tris-HCl buffer, pH 7.4 (120 mM), and microsomal protein (2.5 mg.). All exposures were at 37°. At the indicated times, 0.05 ml. portions were transferred to tubes for adenosine-triphosphatase assay in a final volume of 1 ml. After incubation with ATP, the reaction was stopped with 5% trichloroacetic acid (or, in Expts. 1 and 2, with iodoacetate; see the Experimental section), and samples were chromatographed as described in the Experimental section.

Expt. no.	Enzyme treatment	Adenosine-triphosphatase activity ($\mu\text{moles of inorganic phosphate liberated/mg. of protein/hr.}$)							
		Without NaCl Time of exposure (min.)				With NaCl (100 mM) Time of exposure (min.)			
		0	15	30	60	0	15	30	60
1	Neuraminidase	37	28	22	15	55	41	32	24
	None	41	30	23	18	54	41	35	26
2	Neuraminidase	24	18	16	—	50	37	35	—
	None	23	20	17	—	45	40	35	—
3	Phospholipase A (heated)	31	—	25	12	36	—	33	18
	Phospholipase A (unheated)	31	—	25	12	33	—	31	17
	None	32	—	26	17	37	—	36	20
4	Phospholipase C	—	—	—	—	36	—	9	8
	None	—	—	—	—	36	—	27	25
5	Phospholipase C (5 $\mu\text{g.}$)	17	12	7	—	23	19	12	—
	Phospholipase C (100 $\mu\text{g.}$)	19	9	7	—	24	14	12	—
	None	21	18	14	—	23	23	20	—

effective, although some neuraminic acid-containing ganglioside remained attached to the microsomes even after the treatment.

The preparation of phospholipase C used in the present experiments was only partially purified and was likely to contain other enzymes. However, for its inhibitory effect Ca^{2+} ions were required; also, inhibition was prevented by preincubating the enzyme with an amount of α -antitoxin which lowered the amount of acid-soluble phosphate split (Table 5). The three antisera, which were prepared from filtrates of different types of *C. welchii*, differed in content of titrated antibody and probably in amounts of other antibodies as well (I. Batty, personal communication; see the Experimental section). Hence, correlation of the prevention of the phospholipase C effect on adenosine triphosphatase, with the titre of α -antitoxin, as well as with decrease in the amount of microsomal acid-soluble phosphate split, is considered to indicate strongly that the inhibitory effect on adenosine triphosphatase is due to the α -toxin. The inhibition might be secondary either to breakdown of membrane phospholipids necessary for full enzyme activity, or to inhibition by released phosphorylcholine. However, incubation of microsomes containing 50 $\mu\text{g.}$ of protein with 0.2 μmole of phosphorylcholine, or about 20 times the amount

released by phospholipase C in 30 min., failed to inhibit significantly their adenosine-triphosphatase activity.

DISCUSSION

In the present study of the microsomal adenosine triphosphatase, structural factors have proved important in determining its behaviour.

(1) This was shown first by the unextracted enzyme in the initial microsomal suspensions. Their hydrolysis of ATP in electrolyte solutions decayed with a time-course similar to that of alteration in physical structure as manifested by light-scattering changes. Blount, Rayner, Schmidt-Nielsen & Tosteson (1963) noted aggregation of fragments from sheep-erythrocyte membranes, when suspended in electrolyte solutions. Such aggregation may result from charge effects that interfere with repulsive forces between microsomal particles, and also modify access of the (ionized) substrate. That inactivation was slower when Na^+ ions replaced other cations may be related to Na^+ ion-adenosine triphosphatase being the preferred substrate of the enzyme.

(2) Enzymic attack of microsomal constituents suggested that membrane lipids, especially lecithin, play a role in maintaining the full activity of the system. Since removal of one fatty acid from

Table 5. *Prevention of phospholipase C inhibition of microsomal adenosine triphosphatase*

Microsomes were exposed to phospholipase C as follows. Expt. 1: incubation was in the presence or absence of CaCl_2 (10 mM); the medium contained phospholipase C (5 $\mu\text{g.}$), tris-HCl buffer, pH 7.5 (110 mM), and microsomal protein (2.8 mg.); Expt. 2: to incubation medium containing CaCl_2 (10 mM) and tris-HCl buffer, pH 7.4 (100 mM), was added phospholipase C (0.1 mg.) and antisera diluted to contain the stated number of units of α -antitoxin. After 15 min. at room temperature, microsomes containing 2.4 mg. of protein were added. All exposures were at 37° for 30 min. Adenosine-triphosphatase assays were performed on 0.05 ml. portions. Portions (1 ml.) were added to 1 ml. of 5% trichloroacetic acid for determination of acid-soluble phosphorus.

Expt. no.	Additions to incubation media	Adenosine-triphosphatase activity ($\mu\text{moles of inorganic phosphate liberated/mg. of protein/hr.}$)		Acid-soluble P (total $\mu\text{g.}$)
		Without NaCl	With NaCl (100 mM)	
1	CaCl_2 (10 mM)	20	26	—
	CaCl_2 (10 mM) + phospholipase C	14	19	—
	None	16	35	—
	Phospholipase C	16	36	—
2	Phospholipase C	11	16	0.78
	Phospholipase C + antiserum K 3674 (0.24 unit)	10	15	0.66
	Phospholipase C + antiserum K 3736 (0.24 unit)	11	16	0.67
	Phospholipase C + antiserum K 3663 (0.24 unit)	11	17	0.58
	Phospholipase C + antiserum K 3674 (0.75 unit)	18	26	0.30
	Phospholipase C + antiserum K 3736 (0.75 unit)	16	22	0.38
	Phospholipase C + antiserum K 3663 (0.75 unit)	20	26	0.33
	None	23	28	0.25

the lecithin molecule by phospholipase A did not diminish adenosine-triphosphatase activity, the polar phosphorylcholine moiety is presumably the part of the molecule that must remain in position for maximum adenosine-triphosphatase activity. The Mg^{2+} ion-activated adenosine triphosphatase of muscle (Kielley & Meyerhof, 1950) and muscle-relaxing factor (Ebashi, 1958) are inactivated by phospholipase C, and Schatzmann (1962) found similar effects with erythrocyte membranes. As suggested by Schatzmann (1962), the membrane lipids might stabilize the enzyme protein folding or even play a role in fixing substrate to the active site. Since, however, stimulation by Na^+ ions was evident even after prolonged incubation with phospholipase C, this property must be independent of the presence of the phosphorylcholine group.

(3) The most striking finding in this study is the marked activation of the Na^+ ion-stimulated adenosine triphosphatase by non-ionic detergents. A number of compounds have been described that inhibit similar systems, and protamine has been noted to substitute partly for Na^+ ions as an activator of the system (Schwartz *et al.* 1962). The detergents, however, specifically activate the Na^+ ion-stimulated enzyme and are thus distinct in their action from protamine. The influence of detergent chain length both on solubilization and on adenosine-triphosphatase activation was impressive. Roodyn (1962) noted maximum solubilization of mitochondria with Triton X, which is a

polyoxyethylene ether with n 9–10; much less solubilization occurred with compounds with longer hydrophilic chains. Elworthy & Macfarlane (1963) have reported a number of characteristics of non-ionic detergents that depend on the length of the polyoxyethylene chain. Compounds with longer hydrophilic chains exhibit more hydration and stability in aqueous medium, more symmetry and curling of hydrophilic chains, and smaller micellar size. Characteristics such as these may be important for adenosine-triphosphatase activation as well as for solubilization.

Enzyme activation occurs with concentrations of detergent which are suboptimum for maximum solubilization. Also, separation of the deposit after treatment with detergent showed that the specific activity of non-solubilized microsomes at these concentrations is increased, emphasizing that solubilization is not necessary for stimulation. The detergent evidently penetrates the lipid-water interface of the membrane before micelle formation and solubilization. In this manner, the relationship of enzyme to membrane is probably altered, and it is presumably this alteration that results in augmented enzymic activity. Part of the activation might be due to prolongation of an initial rapid rate of hydrolysis by preventing the usual fall-off in reaction rate. However, the rate in the presence of detergent was greater than the initial rates of untreated microsomes, and linearity was found also when detergent but no sodium chloride was added to the reaction mixture, when the

detergent did not activate. Interaction between detergent and the enzyme protein itself might occur, since other detergents have been shown to alter the structure of a variety of proteins, presumably by interaction with the hydrophobic interior, with subsequent increase in the α -helical conformation of the protein (Jirgensons, 1961, 1952; Meyer & Kauzmann, 1962). Astrup & Alkjaersig (1951) found that quaternary detergents activated trypsin and plasmin present already in molecular solution. These examples, however, have all concerned ionic surface-active agents and soluble proteins, and in these respects differ from the present adenosine-triphosphatase activation.

Another possible explanation for the detergent activation would be release from a normally inhibitory system. In a proposed mechanism of cation transport (McIlwain, 1962*a*, 1963) the adenosine triphosphatase is pictured as inhibited by acidic components of the membrane in the resting state; and Na^+ ions, during their transport, as disinhibiting the enzyme. The non-ionic detergents might then effect a similar disinhibition by insertion of molecules among those of the membrane. The stimulation of adenosine triphosphatase by Na^+ ions was, however, retained after treatment of microsomes with neuraminidase, and was increased, even after solubilization, with non-ionic detergents. Stimulation by Na^+ ions must then be a property either of the enzyme itself or of the enzyme in persistent association with a membrane component. As suggested by McIlwain (1963), acidic groups of the protein itself normally covering the membrane surface might act in the proposed manner. If stimulation by Na^+ ions indicates the degree to which the adenosine triphosphatase is normally coupled to the transport of Na^+ ions, this coupling is increased by the detergents to a degree beyond that obtaining in the microsomal membrane fragments. This may, however, represent only a reimposition of a coupling existing in the intact cell.

The increased activity in the presence of non-ionic detergents, being manifested only with Na^+ ions in the reaction mixture, results in preparations whose adenosine triphosphatase is more potently stimulated by Na^+ ions and inhibited by ouabain. No increase in affinity for Na^+ or K^+ ions of either enzyme or microsomes could be demonstrated, but the detergents led to some loss of cation-binding by the microsomal fraction, and this could explain the smaller activation of the enzyme in the absence of Na^+ ions. Ratios of Na^+ ion-stimulated to non-stimulated adenosine triphosphatase higher than 2-4 have been obtained by Skou (1962), who homogenized whole rabbit brain in EDTA and deoxycholate, which also diminished the activity without Na^+ and K^+ ions present in the medium. On the

other hand, Schwartz *et al.* (1962), by raising the concentration of ATP, abolished the activation by Na^+ ions of microsomal adenosine triphosphatase from guinea-pig brain. These variations in the proportion of enzyme activity that is stimulated by Na^+ ions emphasize the difficulty in determining the amount of activity that is actually important in the transport of Na^+ and K^+ ions. Bonting & Caravaggio (1963) calculated the ratios of adenosine-triphosphatase activity to the flux of Na^+ and K^+ ions for five tissues, utilizing adenosine-triphosphatase values from whole-tissue homogenates and placing most emphasis on the amount of activity dependent on the presence of Na^+ or K^+ ions, or inhibited by ouabain. Since the proportion of such activity seems to depend on the method of assay and of preparation, uncertainty must remain as to just which activity is pertinent in whole tissue. It is possible that a considerable amount of adenosine-triphosphatase activity measured in the absence of Na^+ ions under usual assay conditions may require Na^+ ions for function *in vivo*, and may be part of the ion-transport adenosine triphosphatase. In this case, the ratios between tissue adenosine triphosphatase and transport of Na^+ ions would be diminished from the values of 1.8-3.0 Na^+ ions/'high-energy' phosphate group computed by Bonting & Caravaggio (1963).

SUMMARY

1. Several surface-active agents, ionic and non-ionic, were found to solubilize and to influence the activity of the Na^+ ion-stimulated adenosine triphosphatase of cerebral microsomes. The cationic detergent cetyltrimethylammonium bromide abolished stimulation by Na^+ ions and inhibited enzyme activity. Sodium deoxycholate, an anionic surface-active agent, solubilized 15% of enzyme activity and inhibited the adenosine triphosphatase when measured in the absence of Na^+ ions. The non-ionic detergents digitonin and Lubrol W solubilized 40-50% and in addition activated the adenosine triphosphatase, especially in the presence of sodium chloride. This activation was abolished by ouabain. Rates reached 164 μ moles of inorganic phosphate liberated/mg. of protein/hr., or 2-3 times the rates with untreated microsomes. Activation occurred at concentrations of detergent that were lower than those optimum for solubilization.

2. Solubilization and activation by a series of polyoxyethylene ethers, homologues of Lubrol W, depended on the length of the polyoxyethylene chain. The homologue of *n* average 10 solubilized up to 90% of the adenosine-triphosphatase activity.

3. The adenosine-triphosphatase activity of untreated microsomes exhibited a non-linear time-course. This fall-off in reaction velocity appeared to

result from a change of physical structure of microsomes in electrolyte solutions. After exposure to non-ionic detergents the time-course became linear for 20–30 min.

4. The microsomal adenosine triphosphatase was inhibited by phospholipase C from *Clostridium welchii* which removes phosphorylcholine from lecithin. This effect was prevented by antisera to *C. welchii* and by omitting Ca^{2+} ions from the medium. Neither phospholipase A nor neuraminidase significantly altered the adenosine-triphosphatase activity.

5. These findings indicate that structural factors are important in determining the behaviour of the Na^+ ion-activated adenosine triphosphatase.

P.D.S. was supported by Special Fellowship BT-942 from the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service, during the course of this work, and H.F.B. by a grant from the Medical Research Council. We are grateful to Dr A. Spinks and Dr I. Batty for gifts of materials.

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Constituents of a Microsomal Fraction from the Mammalian Brain: their Solubilization, especially by Detergents

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(Received 14 November 1963)

Many characteristics of neural systems have been ascribed to membrane phenomena, and, on mechanical disruption of cerebral tissues, their cellular membranes appear in microsomal fractions (Toschi, 1959; Hanzon & Toschi, 1959; Wherrett &

McIlwain, 1962). These fractions, like the central nervous system itself, are rich in cholesterol and phospholipids, and also carry appreciable quantities of glycolipids and proteins including specific enzymes such as cholinesterase (Toschi, 1959) and