for allowing separation of its constituents. These reagents are likely to be specific for amino groups, and the aminoacyl bonds they form (Dixon & Perham, 1968) appeared sufficiently labile to allow their hydrolysis under conditions not deleterious to Na,K-ATPase activity.

Microsomal suspensions were prepared from frozen ox cerebral cortex essentially by the procedure of Schwartz, Bachelard & McIlwain (1962) and then treated with an NaI reagent (Nakao, Tashima, Nagano & Nakao, 1965). Determination of amino groups in this preparation with 2,4,6-trinitrobenzenesulphonic acid (Habeeb, 1966) showed that lipid amino groups comprise about 55% of the total. Reaction with methylmaleic anhydride at pH 8 and 20°C causes a loss of Na,K-ATPase activity that is 50% complete when about 5% of the amino groups have been blocked, partial protection against inactivation being afforded by ATP. An 11-fold molar excess of methylmaleic anhydride acylates 90% of the total amino groups, and centrifugation of this acylated material at 150000g for 1 h gives a clear supernatant containing 30% of the protein and 5% of the phospholipid. Dimethylmaleic anhydride at pH 8.5 acylates the enzyme preparation less effectively than does methylmaleic anhydride at pH 8, but loss of Na,K-ATPase activity correlates similarly with the extent of acylation. No more than 43% of the total amino groups are dimethylmaleylated even when the anhydride is in 20- or 30-fold molar excess; centrifugation of this material gives a clear supernatant containing 45% of the protein and less than 10% of the phospholipid.

Regeneration of amino groups from the methylmaleylated material takes place slowly at pH 6 and 20°C, but Na,K-ATPase activity of unmodified material is lost to an extent precluding restoration of enzyme activity. Dimethylmaleyl groups are readily removed at pH 7 and there is partial restoration of Na,K-ATPase activity to the deacylated material provided that not more than 20% of the amino groups were initially acylated.

This investigation was supported by the Research Fund of the Bethlem Royal Hospital and Maudsley Hospital.

Dixon, H. B. F. & Perham, R. N. (1968). *Biochem. J.* **109**, 312.

Habeeb, A. F. S. A. (1966). *Analyt. Biochem.* **14**, 328.

Nakao, T., Tashima, Y., Nagano, K. & Nakao, M. (1965). *Biochem. biophys. Res. Commun.* **19**, 755.

Schwartz, A., Bachelard, H. S. & McIlwain, H. (1962). *Biochem. J.* **84**, 626.

Sodium-plus-Potassium Ion-Activated Adenosine Triphosphatase in a Heavy-Membrane Fraction Isolated from Rat Skeletal Muscle

By A. S. F. ASH and A. SCHWARTZ. (*Department of Pharmacology, Royal Free Hospital School of Medicine, University of London, London W.C.1, U.K., and Division of Myocardial Biology, Baylor College of Medicine, Houston, Tex. 77025, U.S.A.*)

An Mg^{2+} -dependent $Na^+ + K^+$ -activated adenosine triphosphatase, participating in active cation transport, has been isolated from a variety of tissues including cardiac (Matsui & Schwartz, 1966) and skeletal muscle (Samaha & Gergely, 1965). The enzyme activity is generally associated with membrane fractions derived from the endoplasmic reticulum and the plasma membrane (Skou, 1965). Current techniques for obtaining this adenosine triphosphatase involve treatment with deoxycholate. However, the results obtained with rat skeletal muscle were variable and the activity of the enzyme was low (Rogus, Price & Zierler, 1969). A method is now described for obtaining this $Na^+ + K^+$ -activated enzyme with high and reproducible specific activity. The enzyme was isolated at 0–4°C from a heavy-membrane fraction, without deoxycholate treatment, after solubilization of actomyosin.

Rat skeletal muscle was homogenized in 0.25 M-sucrose–1 mM-tris–EDTA buffer, pH 7.8, with an Ultra-Turrax tissue processor ($3 \times 3s$, 20000 rev./min). A precipitate collected at 600g (10 min) was treated with 0.6 M-KCl in 20 mM-tris-maleate buffer, pH 7.0, to solubilize actomyosin, rehomogenized and centrifuged again at 650g (15 min). The precipitate was twice washed in the sucrose–EDTA medium, collected at 10000g (15 min) and suspended in 1 mM-tris–EDTA buffer, pH 7.0, to give a protein concentration of 10 mg/ml.

An increase in the Mg^{2+} -dependent adenosine triphosphatase activity from 19.7 ± 1.18 to $28.7 \pm 1.16 \mu\text{mol of } P_i/\text{h per mg of protein}$, produced by addition of 100 mM- Na^+ with 10 mM- K^+ , was inhibited by ouabain ($K_i \ 8 \times 10^{-5} \text{ M}$). Total adenosine triphosphatase activity declined with time, but the ouabain-sensitive release of P_i from ATP (optimum pH 7.4) was linear over 5 min. The $Na^+ + K^+$ -stimulated activity was not enhanced by NaI treatment. The preparation was stable for several hours at 0°C, but activity declined overnight at 0°C or –20°C.

Inhibition of the $Na^+ + K^+$ -stimulated enzyme by ouabain (0.5 mM) was increased by 10% on addition of a low concentration of oligomycin (0.5 $\mu\text{g}/\text{mg}$ of protein); this suggests that some of the enzyme activity was of mitochondrial origin. At a higher concentration (5 $\mu\text{g}/\text{mg}$ of protein) oligomycin alone

inhibited the adenosine triphosphatase more effectively than ouabain. Oligomycin has an ouabain-like action on cell membranes (Whittam, Wheeler & Blake, 1964) and may therefore be considered as an alternative inhibitor for the characterization of $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase, particularly in the rat, which has a low sensitivity to cardiac glycosides (Allen & Schwartz, 1969).

- Allen, J. C. & Schwartz, A. (1969). *J. Pharmac. exp. Ther.* **168**, 42.
 Matsui, H. & Schwartz, A. (1966). *Biochim. biophys. Acta*, **128**, 380.
 Rogus, E., Price, T. & Zierler, K. L. (1969). *J. gen. Physiol.* **54**, 188.
 Samaha, F. J. & Gergely, J. (1965). *Archs Biochem. Biophys.* **109**, 76.
 Skou, J. C. (1965). *Physiol. Rev.* **45**, 596.
 Whittam, R., Wheeler, K. P. & Blake, A. (1964). *Nature, Lond.*, **203**, 720.

The Preparation of Acetylcholinesterase from Human Erythrocytes

By D. L. WRIGHT and D. T. PLUMMER. (*Department of Biochemistry, Chelsea College of Science and Technology, London S.W.3, U.K.*)

Acetylcholinesterase is firmly bound to the erythrocyte membrane (Augustinsson, 1948) so that it is desirable in any investigation of its molecular properties to bring the enzyme into solution. Gordon & Rutland (1967) used ultrasonic vibration to 'solubilize' the acetylcholinesterase of ox erythrocytes, but the adaptation of this method to a large-scale preparation might prove difficult; also, treatment with ultrasound could change the electrophoretic properties of the enzyme (Dubbs, 1966). We now report a method using a mixture of KCl and the non-ionic detergent Triton X-100 to prepare soluble acetylcholinesterase.

Human erythrocytes were washed three times in saline, lysed in 6 vol. of water and after three washings in distilled water, stroma were suspended in 8 vol. of KCl-Triton X-100. A range of concentrations of KCl and Triton X-100 was examined to determine the optimum conditions for solubilization. The enzyme was considered to be soluble if it did not sediment when subjected to 100 000 *g* for 1 h. Over 90% of the enzyme was soluble in a mixture containing 1.2 M-KCl and 1% Triton X-100.

Addition of ammonium sulphate to 60% saturation precipitated about 70% of the soluble enzyme activity. The precipitate was soluble in saline and had a specific activity of 0.5 $\mu\text{mol}/\text{min}$ per mg of protein, representing about a 50-fold purification with respect to the haemolysate. The Michaelis constant of the enzyme at this stage was 75 μM compared with 69 μM for the crude haemolysate.

Electrophoresis of the partially purified preparation on 6% polyacrylamide gel at pH 8.1 (Smith, 1968) showed between one and three bands of esterase activity with α -naphthyl acetate as substrate (Paul & Fottrell, 1961): all the esterase bands were sensitive to eserine. Whether the occurrence of more than one band represents several forms of the enzyme is under investigation.

We are grateful to the Wellcome Trust for providing equipment and running expenses for this project.

- Augustinsson, K. (1948). *Acta physiol. scand.* **15**, suppl. 52.
 Dubbs, C. A. (1966). *Clin. Chem.* **12**, 181.
 Gordon, J. J. & Rutland, J. P. (1967). *Nature, Lond.*, **214**, 850.
 Paul, J. & Fottrell, P. (1961). *Biochem. J.* **78**, 418.
 Smith, I. (1968). *Chromatographic and Electrophoretic Techniques*, vol. 2, p. 403. London: W. Heinemann Ltd.

Isolation of Nicotinamide-Adenine Dinucleotide-Cytochrome *b*₅ Oxidoreductase from Rat Liver Microsomes

P. J. ST LOUIS, J. R. SARGENT and PATRICIA A. BLAIR. (*Department of Biochemistry, University of Aberdeen, Aberdeen AB1 3RA, U.K.*)

Strittmatter & Velick (1957) isolated NADH-cytochrome *b*₅ oxidoreductase (EC 1.6.2.2) from calf liver microsomes after digestion with snake venom at pH 5.7. Takesue & Omura (1968), however, have reported that release of the oxidoreductase is due not to snake venom but to lysosomes contaminating the microsomes. The present communication confirms the latter result and presents evidence that the active lysosomal principle is an acid proteinase. A simple method for isolating the oxidoreductase in a pure state is also presented.

Treatment of rat liver microsomes with snake venom, steapsin, pepsin, trypsin or chymotrypsin failed to release appreciably active oxidoreductase. A lysosomal fraction of rat liver sedimenting between 5×10^4 and 8×10^4 *g*-min was active in releasing the microsomal oxidoreductase into the supernatant phase after centrifuging at 9.45×10^6 *g*-min. Essentially quantitative release of the oxidoreductase occurred with equal weights of microsomes and lysosomes on a protein basis were incubated at 37°C for 120 min in 0.1 M-tris-maleate buffer, pH 5.7. A three- to four-fold activation of the enzyme was consistently observed after its release.

The lysosomal fraction was fractionated with Triton X-100 to yield a high-speed supernatant, which, when extracted with pentanol and chromatographed on Sephadex G-200, yielded an acid proteinase fraction 20–25 times enriched over the starting lysosome fraction. The proteinase activity