Differences have been found previously in the metabolic products of thyroxine in hepatic and extrahepatic tissues of the dog (Flock & Bollman, 1960). Thus dogs with livers readily deiodinate thyroxine in the 3′- or 5′-positions and excrete large amounts of radioactive iodide in the urine. Dogs without livers deiodinate thyroxine from the 3- and 5-positions to a greater extent than from the 3′- and 5′-positions. Conjugates of 3,3′,5′-tri-iodothyronine and 3,3′-di-iodothyronine accumulate in plasma and urine of the hepatectomized dog, but only small amounts of radioactive iodide are excreted in the urine. Possibly prolonged feeding of thiouracil in the rat causes inhibition of deiodination of thyroxine by the liver, with subsequent accumulation of partially deiodinated products of thyroxine formed by extrahepatic tissues.

SUMMARY

1. The rat conjugates thyroxine chiefly with glucuronic acid and to a very small extent also with sulphuric acid and with an unidentified substance.

2. After the administration of radioactive thyroxine the rat excretes larger amounts of thyroxine glucuronide and smaller amounts of 3,3′,5′-tri-iodothyronine glucuronide in the bile than the dog does.

3. Previous studies showing that deiodination of thyroxine from the 3′- or 5′-position is decreased in rats after receiving thiouracil have been confirmed.

4. Deiodination from the 3- or 5-position of thyroxine is increased in rats given a diet containing 0·1% of thiouracil for 28 days or longer. This may account in part for the marked reduction in calorigenic activity of thyroxine administered to thiouracil-treated rats.

The L-thyroxine, L-tri-iodothyronine and tetraiodothyroacetic acid used as markers on the chromatograms were gifts from Dr A. E. Heming of Smith, Kline and French Laboratories, Philadelphia; the 3,3′,5′-tri-iodo-DL-thyronine and 3,3′-di-iodo-DL-thyronine were gifts from Dr R. L. Kroc of the Warner–Lambert Research Institute, Morris Plains, New Jersey, research affiliate of Warner–Chilcott Laboratories. We wish also to thank Mrs Jauneita Ogg and Mr E. Van Hook for valuable technical assistance. This investigation was supported in part by Research Grant A-2780 from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service.

REFERENCES

Andik, I., Balogh, L. & Donhoffer, S. (1949). *Experientia*, **5**, 249.
Astwood, E. B. (1945). *Harvey Lect.* **40**, 195.
Closon, J. (1959). Ph.D. Thesis: Faculté des Sciences, Paris.
Cruchaud, S., Vannotti, A., Mahaim, C. & Deckelmann, J. (1955). *Lancet*, ii, 906.
Escobar del Rey, F. & Morreale de Escobar, G. (1961). *Endocrinology*, **69**, 456.
Flock, E. V. & Bollman, J. L. (1960). *Fed. Proc.* **19**, 176.
Flock, E. V. & Bollman, J. L. (1961). *Biochem. J.* **81**, 18P.
Flock, E. V., Bollman, J. L. & Grindlay, J. H. (1960). *Endocrinology*, **67**, 419.
Flock, E. V., Bollman, J. L., Grindlay, J. H. & Stobie, G. H. (1961). *Endocrinology*, **69**, 626.
Franklin, A. L., Lerner, S. R. & Chaikoff, I. L. (1944). *Endocrinology*, **34**, 265.
Hogness, J. R., Wong, T. & Williams, R. H. (1954). *Metabolism*, **3**, 510.
Jones, S. L. & Van Middlesworth, L. (1960). *Endocrinology*, **67**, 855.
Maclagan, N. F. & Reid, D. (1957). *Ciba Found. Coll.: Endocrinology*, p. 190.
Roche, J., Michel, R., Closon, J. & Michel, O. (1959). *Biochim. biophys. Acta*, **33**, 461.
Stasilli, N. R., Kroc, R. L. & Edlin, R. (1960). *Endocrinology*, **66**, 872.
Taurog, A., Briggs, F. N. & Chaikoff, I. L. (1952). *J. biol. Chem.* **194**, 655.
VanArsdel, P. P., jun. & Williams, R. H. (1956). *Amer. J. Physiol.* **186**, 440.

Biochem. J. (1962) **84**, 626

The Sodium-Stimulated Adenosine-Triphosphatase Activity and other Properties of Cerebral Microsomal Fractions and Subfractions

BY A. SCHWARTZ, H. S. BACHELARD AND H. McILWAIN
Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5

(*Received* 2 *April* 1962)

The metal activation and the likely complexity of cerebral enzymes forming inorganic phosphate and adenosine diphosphate from adenosine triphosphate were examined by Gore (1952) and by Lowry, Roberts, Wu, Hixon & Crawford (1954). Subsequently, acceleration by Na^+ ions was found to distinguish the adenosine-triphosphatase activity of cerebral microsomal fractions from that of cerebral mitochondria (Deul & McIlwain, 1961*a*, *b*); both enzymes required potassium and magnesium

Table 1. *Primary fractions obtained by differential centrifuging of dispersions of cerebral cortex*

Tissue was dispersed in 0·32M-sucrose as described in the text; for washing, the deposits were thoroughly dispersed in a homogenizer with 0·32M-sucrose before centrifuging again.

Description of deposited material	Conditions of centrifuging: Material	*g*	Time (min.)	Washing
N, nuclear	Initial dispersion	600	10	1 (initial vol.)
Mt_1, mitochondrial	Supernatant and washings from N	10 000	15	2 (0·5 initial vol.)
	Washings from Mt_1	10 000	15	None
Ms, microsomal	Supernatants from Mt_1	20 000	60	1 (initial vol.)
	Supernatants from Mt_2	20 000	60	None
Mt_2, second mitochondrial	Ms	10 000	15	None

salts. The cerebral microsomal enzyme was thus recognized as akin to that of fine particles from crab nerve, considered by Skou (1957, 1960) to be associated with cation transport, as has been indicated also by Järnefelt (1961) and by Skou (1962).

The microsomal fractions consist, however, of two main components (Toschi, 1959; Hanzon & Toschi, 1960): ribonucleic acid granules and membrane structures. As the ribonucleic acid granules may consume adenosine triphosphate by processes concerned with protein synthesis rather than ion transport, it was considered valuable to attempt to localize the sodium-activated enzyme within the microsomes, and the preparation and subfractionation of cerebral microsomal material from this point of view are now described. During the preparation and subfractionation, the ribonucleic acid, cholesterol, succinic dehydrogenase and cholinesterase of the preparations have been determined.

To further understand the sodium-activated adenosine triphosphatase, attempts have been made to obtain it in solution and to find how its activity is affected by substances that modify ion movements in intact cerebral tissues. Such substances include ouabain (Whittam, 1961; Schwartz, 1962; Aldridge, 1962), basic proteins, gangliosides and suramin (Thomson & McIlwain, 1961; McIlwain, Woodman & Cummins, 1961). Also, because they affect the adenosine-triphosphatase activity of other systems, 2,4,6-trinitrobenzenesulphonate (Kubo, Tokura & Tonomura, 1960) and 2,4-dinitrophenol (Perry & Chappell, 1957) have also been examined.

EXPERIMENTAL

Tissue dispersion and fractionation

Initial dispersions. Guinea pigs were struck on the neck, exsanguinated, the cerebral hemispheres taken and their white matter was removed. The grey matter, usually 1·8 g./animal, was placed within 3 min. of death in an ice-cold Teflon homogenizer (A. H. Thomas Co.; clearance 0·13–0·18 mm.) and in batches of 3·5–6·0 g. was immediately ground in 9 vol. of 0·32M-sucrose that had been adjusted to pH 7·4 with KOH. Grinding involved 10 passages of the pestle, rotating at 2300 rev./min., during 20 sec., with intermediate periods of cooling in ice.

A few preparations were made from ox brain, collected promptly after death at a slaughterhouse and placed in dry plastic bags surrounded by ice. In a cold room, the dura and adhering blood were removed and grey matter from the cerebral cortex was snipped off with scissors, suspended in 5 vol. of sucrose in the above-mentioned homogenizer, and batches of 50 ml. were passed through an Emanuel & Chaikoff (1957) piston-press homogenizer, orifice 25 μ, at 3° (see Deul & McIlwain, 1961*b*).

Centrifuging. Forces have been expressed as average *g* values calculated to the centres of the tubes. For forces up to 20 000*g*, an angle head of the Measuring and Scientific Equipment Ltd. 17 000 centrifuge was used, and for greater forces the no. 40 head of the Spinco model L ultracentrifuge (except that its swing-out head SW 39 was used for density-gradient centrifuging).

Preparation of the primary fractions from guinea-pig cerebral cortex is summarized in Table 1 and is based on previous experience with cerebral tissues (McIlwain & Rodnight, 1962); the following points are to be noted in relation to the earlier study of Deul & McIlwain (1961*b*). The nuclear fraction is prepared at the same speed, now stated as an average *g* value; however, it contains less protein and adenosine triphosphatase and this is attributed to the use of the present homogenizer rather than the piston-press homogenizer used previously. The first mitocondrial fraction combines the two of Deul & McIlwain (1961*b*), but is washed more thoroughly; the washings are centrifuged again and deposited material is added to fraction Mt_1. The microsomal material is prepared at a slower speed; by resuspending it and centrifuging as indicated, mitochondria are removed (see below) as fraction Mt_2.

For density-gradient centrifuging, 5 ml. tubes were prepared with 1 ml. each of 0·6, 0·9, 1·2 and 1·5M-sucrose. The microsomal suspension in either 0·32 or 0·6M-sucrose was placed above the gradient. Centrifuging at $10^5 g$ for 3 hr. followed, after which specimens were withdrawn with fine pipettes and were diluted to a sucrose content of 0·3M. Control experiments showed that exposure of microsomal samples in the stronger sucrose solutions for the duration of centrifuging did not affect the determinations made.

Keeping. Fractions were kept in the 0·32M-sucrose in which they were prepared, at 0–3° unless otherwise specified. Some were kept as samples of 0·5 ml. in numerous tubes closed with plastic sheet and in a deep-freeze at −20° to −27°.

Microscopic examination. Fractions were examined in 0·32 M-sucrose with a phase-contrast microscope, and also after staining with Janus green B (G. T. Gurr Ltd.) as follows. A drop of an ethanolic 1% solution of the dye was spread on a microscope slide and allowed to dry, and one drop of a suitable dilution of a tissue preparation in 0·32 M-sucrose (e.g. 1:10) and a coverslip were added. Mitochondria were first bluish green but changed within 0·5–1 min. through colourless to red (Showacre & Du Buy, 1955).

Reagents and analyses

The protamine used was, unless otherwise specified, a specimen of clupeine sulphate (L. Light and Co. Ltd.) containing: total N, 24·7%; sulphate S, 6·36%; equiv. wt. of the sulphate based on sulphate, 251 (compare Callanan, Carroll & Mitchell, 1957; Carroll, Callanan & Saroff, 1959). In view of the experiments of Table 5, the specimen was examined for sodium and potassium, and any present found to be less than 0·0025 equiv./equiv. of protamine. We are indebted to Mr D. A. Booth for the specimen of gangliosides from ox brain, which was prepared according to McIlwain (1961) and yielded 26·4% of *N*-acetylneuraminic acid (Booth, 1962). 2,4,6-Trinitrobenzenesulphonic acid was kindly given by Dr R. Hull, Imperial Chemical Industries Ltd., Pharmaceuticals Division; the firm also supplied suramin.

Tris–adenosine triphosphate. Two batches of 2·5 g. of Dowex 50 resin (H^+ form) were washed successively with 50 ml. of N-HCl, water, aq. N-NH_3 soln., water, N-HCl and water in beakers and then with water on a funnel by suction until the washings were neutral. The batches were then transferred to 15 ml. conical flasks in a cold room; 0·5 g. of disodium adenosine triphosphate (Sigma Chemical Co.) in 2 ml. of water was added to one flask and the contents were stirred for 15 min., filtered by suction and the resin was washed twice with 1 ml. of water. The filtrate was added to the second flask and treated similarly, giving a filtrate that was made to pH 7·4 with M-tris. Water was added to make the solution 15 mM in adenosine triphosphate, based on the extinction at 260 mμ of a dilution. A 0·001 dilution of 15 mM-adenosine triphosphate has *E* 0·230; usually the 0·5 g. of triphosphate gave 50–60 ml. of 15 mM solution (a 85–95% yield), which was found to be 0·1–0·14 mM in sodium and less than 0·1 mM in inorganic phosphate.

Adenosine-triphosphatase assay. Reaction mixtures of 1 ml. were prepared in tubes in ice, containing under standard conditions: 100 mM-tris–HCl, pH 7·4; 100 mM-NaCl; 30 mM-KCl; 3 mM-$MgCl_2$; tris–adenosine triphosphate, 3 mM. They were shaken at 37° for 5 min.; a solution to be assayed was added, shaking continued for 10 min. and the tube was then placed in ice and 1 ml. of 5% (w/v) trichloroacetic acid added. Variants included the addition of further reagents, modifying the order of addition and carrying out the reaction in a larger volume and sampling at intervals to follow the course of reaction. Inorganic phosphate was determined as described by Deul & McIlwain (1961*b*); in this determination it is to be noted that trichloroacetic acid precipitation and centrifuging are required in spite of the presence of the silicotungstate reagent.

Acetylcholinesterase assay. A manometric method similar to that of Aldridge & Johnson (1959) was applied to tissue samples shortly after their preparation; their activity diminished in 1 day at 0° or −20°. The sample, derived from about 0·1 g. of tissue, was included in 4 ml. of solution 130 mM in NaCl, 35 mM in $MgCl_2$ and 31 mM in $NaHCO_3$, in the main compartment of a conical vessel. After equilibration with CO_2+N_2 (5:95) at 37°, acetylcholine chloride to make 13·8 mM was tipped from a side arm and pressure readings were taken each 5 min. for 35 min.

Succinic dehydrogenase. Suspensions of cerebral tissues were found to increase in succinic-dehydrogenase activity on freezing and thawing. To obtain maximal activities preparations were left at −20° overnight, thawed and left refrozen for 24 hr. To the thawed suspension an equal volume of water was added and samples carrying 5–10 mg. of protein were used in a manometric assay based on those of Quastel & Wheatley (1938) and Aldridge & Johnson (1959). The final concentrations of reagents were 25 mM-$NaHCO_3$, 6 mM-$MgCl_2$, 8 mM-KH_2PO_4, 0·8 mM-EDTA, 40 mM-sodium succinate and 17 mM-$K_3Fe(CN)_6$; volume was 3 ml. The ferricyanide remained in a side arm until after equilibration with CO_2+N_2 (5:95), when it was tipped and pressure readings were taken each 5 min. for 35 min.

Protein. This was determined according to Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin as standard. When sucrose was present in the samples analysed it was included in similar concentration in the albumin standards, as it diminished colour formation.

Ribonucleic acid. This was determined by the method of Littlefield, Keller, Gross & Zamecnik (1955) as used by Toschi (1959).

Free cholesterol. We are indebted to Mr H. Bradford for these determinations (Table 3). Samples of an homogenate or subfraction were chosen on the basis of preliminary assays to contain 0·6–1·5 mg. of cholesterol. The sample, already in 1–5 ml. of 0·32 M-sucrose, was shaken vigorously in a glass-stoppered tube for about 20 sec. with 19 ml. of chloroform–methanol (2:1, v/v), with which it formed a single phase carrying a precipitate. After 10 min., the contents of the tube were filtered (sintered glass) and to the filtrate, in a glass-stoppered tube that could be centrifuged, was added 0·2 vol. of 0·1 M-KCl. The tube was shaken and centrifuged at 5000*g* for 5 min.; the upper phase was discarded and the lower phase, in the same tube, was shaken with 0·2 vol. of 'upper-phase mixture' (0·1 M-KCl–methanol–$CHCl_3$, 47:45:3, by vol.). After centrifuging as before the upper phase was again discarded.

The lower phase, 20–90 ml., was evaporated in a small flask in a stream of air from a bath at 50° and the residue dissolved in 1 ml. of light petroleum, b.p. 40–60°, and 3 ml. of acetone was added followed by 0·5 ml. of ethanolic saturated $MgCl_2$. After 5 min. at room temperature the mixture was filtered (sintered glass) and the precipitate washed on the filter with 1 ml. of acetone. Filtrate and washings were made up with acetone to 5 ml. and two 2 ml. samples taken for the remainder of the determination described by McIlwain & Rodnight (1962).

RESULTS

Assay and sodium dependence of the adenosine-triphosphatase activity

The standard-assay conditions (see Experimental section) were based on the investigation by

Deul & McIlwain (1961*b*), which differentiated between mitochondrial and microsomal adenosine triphosphatases. Liberation of inorganic phosphate under these conditions was linear with time for at least 10 and often for 20 min. (Fig. 1), and, when the reactants were mixed in a different order so that the reaction was started by addition of adenosine triphosphate, the rate of reaction was the same as in the standard method. Rates were proportional to quantity of tissue preparation (Fig. 1*B*). They were independent of the concentration of tris buffer, which was examined between 30 and 300 mM.

Dependence on sodium concentration (Fig. 2*A*) indicates the 100 mM-Na of the standard mixture to be sufficient for maximal or almost maximal activation; half-maximal activation is given in these experiments by 15 mM-Na. The standard mixture is at optimum pH, and similar activation by sodium salts, of 1·8- to 3·0-fold, was observed over a wide range of pH values (Fig. 2*B*), within which the activity of a given preparation changed at least fourfold. Activation by sodium salts also remained within this range during changes which took place in the activity of the preparations on keeping at 4° and after freezing (Fig. 3). Immediately on preparation and before freezing, sodium activation, though present, was smaller. These changes were examined as one microsomal pre-

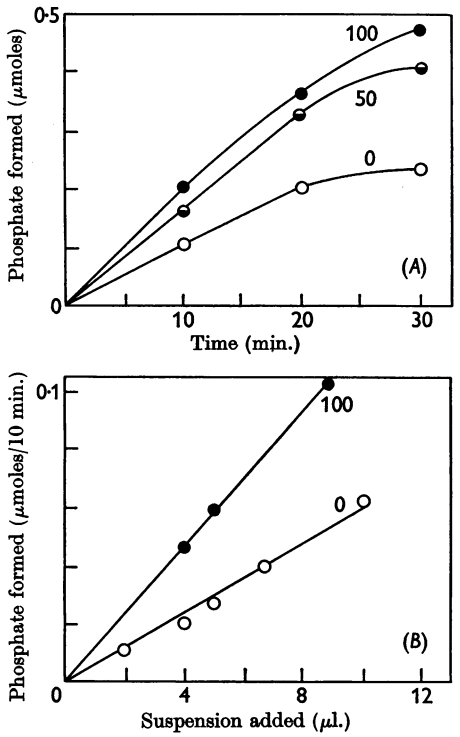

Fig. 1. Adenosine triphosphatase of guinea-pig microsomal fractions kept at −20° for 4 days. (*A*) Course of liberation of inorganic phosphate under standard-assay conditions, but in media with the concentrations of NaCl indicated (mM). (*B*) Liberation under standard conditions but with the quantities of tissue suspension given as abscissae; figures on the curves give NaCl concentration (mM).

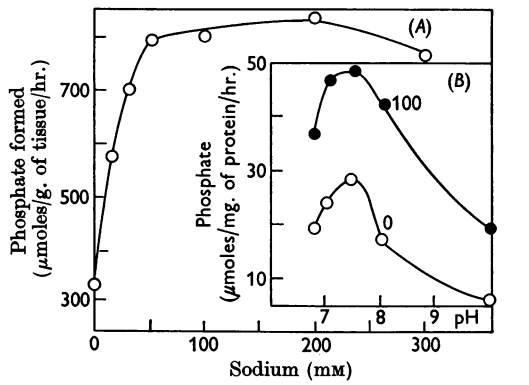

Fig. 2. Dependence of adenosine triphosphatase of guinea-pig microsomal fractions on sodium concentration and on pH, under standard-assay conditions except for the variable stated in abscissae. (*A*) Concentration of the NaCl of the standard mixture was altered as indicated; the fraction had been kept at −20° for 5 days. (*B*) Tris was taken to pH 10 by KOH and to the other pH values by HCl; the fraction had been kept at −20° for 8 days and a similar result was given with a fraction kept for 2 days. Figures on the curves give NaCl concentration (mM).

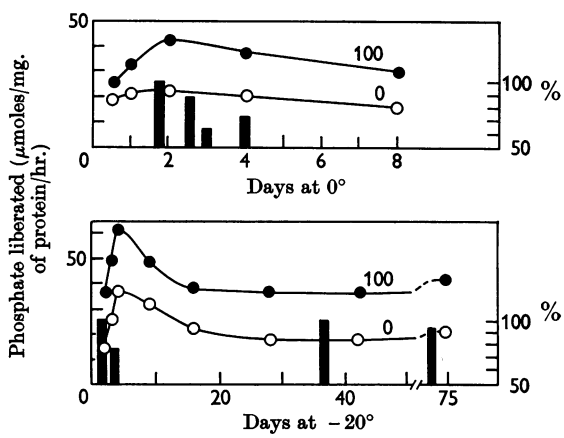

Fig. 3. Change in the adenosine-triphosphatase activity of guinea-pig microsomal fractions kept in 0·32M-sucrose and examined in standard reaction mixtures of the sodium content indicated (mM) by figures on the curves. Vertical bars give the activities observed with 100 mM-Na and 0·3 mg. of ganglioside/ml., as percentages of the values without ganglioside (scale at right-hand side).

paration provided material for several experiments, and the net activity per unit weight of tissue also increased on keeping the suspension in the 0·32 M-sucrose in which it had been prepared. At 0°, activity increased by some 40 % in 2 days and subsequently fell; at −20°, maximal activity was found in 4 days, again followed by diminution, after which activity remained relatively constant.

The extent to which the enzyme was stimulated by sodium salts depended on the concentration of adenosine triphosphate in the reaction mixture. In Fig. 4 reciprocals of reaction velocities, with and without 100 mM-Na, are plotted against reciprocals of adenosine triphosphate concentrations. This is in the form of a Lineweaver–Burk plot; its interpretation in the present instance receives comment in the Discussion. The degree of stimulation is seen to diminish with increase in adenosine triphosphate, and indeed the maximum velocities obtained by extrapolation are independent of the presence of sodium, and with this preparation correspond to 110 μmoles of phosphate/mg. of protein/hr. As display of sodium activation was important to fractionation procedures, an adenosine triphosphate concentration of 3 mM has been retained in the present study. It was adopted initially (Deul & McIlwain, 1961*b*) as it approximated to the average cerebral content of the triphosphate, and had been used in earlier investigations of peripheral nerve (Skou, 1957, 1960).

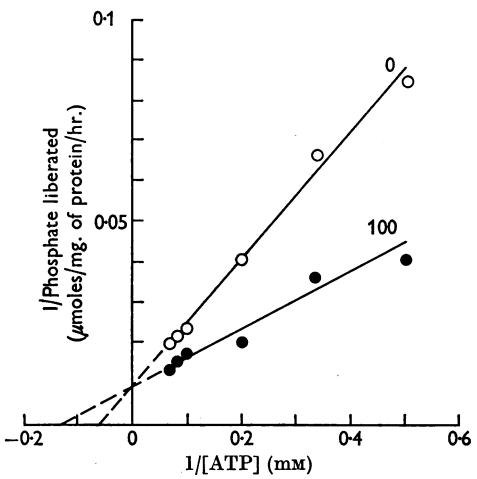

Fig. 4. Dependence of velocity of adenosine-triphosphatase activity on adenosine triphosphate concentration (stated as reciprocals) in standard reaction mixtures with and without 100 mM-Na but with the initial triphosphate concentrations quoted. The guinea-pig microsomal preparation used had been kept 1 day at −20°; a preparation so kept for 46 days gave with 100 mM-Na an apparent K_m of 6·5 mM and a maximum velocity of 71 μmoles/mg. of protein/hr.

In the standard reaction mixture the enzyme utilizes a small proportion only of the adenosine triphosphate present, producing about 0·1 μmole of inorganic phosphate/ml. The effects on the microsomal enzyme of these and rather larger concentrations of the products of the reaction were examined. Inorganic phosphate at 0·2 mM had no effect in the presence of sodium, but in its absence inhibited by 21 ± 8 % (six values, preparations kept 5–180 days). Adenosine diphosphate at 0·3 mM had little or no effect, but higher concentrations were examined in view of its inhibition of a myosin adenosine triphosphatase (Green & Mommaerts, 1954). With 0·8 mM-adenosine diphosphate no action was observed in the absence of sodium, but in its presence there was inhibition by 22 % (three values; preparations kept 3–80 days). With 1·5 mM-adenosine diphosphate it inhibited in the absence of sodium by 11 ± 8 % (five values; preparations kept 5–80 days), and, in its presence, by 31 ± 11 % (same five preparations). Adenosine 5-monophosphate was found to be without effect on the reaction; the enzyme yielded no significant inorganic phosphate from adenosine diphosphate.

Enzyme distribution in tissue fractions

Primary fractions by differential centrifuging. The additional inorganic phosphate formed from adenosine triphosphate by including 100 mM-Na in reaction mixtures initially of less than 0·1 mM-Na was used as a guide to obtaining satisfactory fractionation. That developed after a number of trials is shown in Table 1. The procedure gave three-quarters of the sodium adenosine-triphosphatase activity of the tissues in a single fraction which contained less than one-seventh of the protein (Table 2). It was obtained by modifying the centrifuging sequence frequently used with cerebral dispersions, by repeatedly washing the mitochondrial fraction, and by centrifuging the microsomal fraction at 150 000*g*-min., which removed from it a second mitochondrial fraction. Mitochondrial contamination was appraised by staining (see Experimental section) and by succinic-dehydrogenase assay (Table 3), which showed the microsomal fraction to contain only 3 % of the succinic dehydrogenase of the dispersion. The microsomal fraction was collected at $1{\cdot}2 \times 10^6 g$-min. rather than at the greater forces that have been used and which result in deposition of much more ribonucleic acid (Table 3; see Hanzon & Toschi, 1959; Wherrett & McIlwain, 1962).

These separations indicate a distinctive localization of the sodium-stimulated adenosine-triphosphatase activity: (i) they confirm the previous (Deul & McIlwain, 1961*b*) differentiation from the mitochondrial adenosine triphosphatase which constitutes (Table 2) over half the activity in the

absence of sodium; (ii) they suggest an association with one only of the two main constituents of cerebral microsomal fractions: the membrane structures rather than ribonucleic acid granules (Hanzon & Toschi, 1959). The present fraction Ms carried (Table 3) one-sixth only of the ribonucleic acid of the dispersion. This differentiation was confirmed by acetylcholinesterase determinations (Table 3). Though much of this enzyme occurred in the mitochondrial fraction Mt_1, which had 3·4 times the protein of fraction Ms, the activity per unit protein was much greater in Ms. In particular, the activity per unit protein was 3·3 times as great in Ms as in S, and in electron-microscopic examination Hanzon & Toschi (1959) found cholinesterase associated with membrane structures rather than with ribonucleic acid granules.

Other fractions. (i) The supernatant from fraction Mt_1 (Table 1) was brought to pH 5·3, at which flocculation occurred, by HCl or acetic acid and the separated material was collected, a method used by Gibson, Wilson & Udenfriend (1961) to obtain microsomal material. The deposited material was less active, per unit weight of tissue or per unit protein, than Ms. (ii) Material deposited from the supernatant from Mt_1 by protamine (Hele & Finch, 1960) was similarly less active. (iii) Ox brain, dispersed on a larger scale with a piston-press homogenizer and treated according to the scheme of Table 1, yielded a greater proportion of its protein and adenosine triphosphatase in the nuclear fraction. In (i) to (iii), the microsomal adenosine-triphosphatase activity was approximately doubled by 100 mM-Na added to standard reaction mixtures lacking sodium.

Density-gradient centrifuging. Gradients of sucrose between 0·6 and 1·5 M were used, which differed a little from those of a previous study (Wherrett & McIlwain, 1962) and which gave a broader protein distribution: of the five fractions collected (Fig. 5) the first and last contained about 10%, and the others 20–35%, of protein. The densest fraction, a translucent pellet, was again greatly enriched in ribonucleic acid. Distribution of the adenosine triphosphatase, assayed either with or without sodium activation, differed markedly from that of the ribonucleic acid, for the enzyme was enriched in the middle fraction. In this it resembled cholinesterase (Fig. 5), which Toschi (1959) and Hanzon & Toschi (1960) found to be firmly associated with the membrane structures of cerebral microsomal fractions.

Attempts to solubilize the adenosine triphosphatase. Microsomal fractions Ms deposited at $1·2 \times 10^6 g$-

Table 2. *Adenosine-triphosphatase activities of primary fractions from guinea-pig cerebral cortex*

Fractions were those of Table 1, S being the supernatant from fraction Ms. Assays were carried out under standard conditions (see Experimental section). Mean values from three preparations are quoted, determined after keeping for 2 days at −20°.

		Adenosine triphosphatase				
		Without Na	With Na			
Fraction	Protein (mg./g. of tissue)	(*a*)	(*b*)	(*b*) − (*a*)	Without Na	With Na
		(μmoles/g. of tissue/hr.)			(μmoles/mg. of protein/hr.)	
(Whole dispersion)	103	1283	1685	402	12·3	16·2
N	9·9	59	64	5	9·4	10·2
Mt_1	45·7	685	795	110	15·4	18·0
Mt_2	2·9	51	55	4	17·1	18·4
Ms	13·8	380	708	328	27·6	51·0
S	32·9	189	189	0	5·3	5·3

Table 3. *Properties of primary fractions from guinea-pig cerebral cortex*

Fractions were from the same experiments as those of Table 2, which gives the protein content of the fractions. Analyses are mean values from three experiments, except those of the cholesterol values: that of the nuclear fraction derives from four and the others from two experiments. The enzyme assays (see Experimental section) give values in terms of μmoles of CO_2.

	Ribonucleic acid		Cholesterol		Succinic dehydrogenase		Acetylcholinesterase	
Fraction	μg./g. of tissue	μg./mg. of protein	μmoles/g. of tissue	μm-moles/mg. of protein	μmoles/g. of tissue/hr.	μmoles/mg. of protein/hr.	μmoles/g. of tissue/hr.	μmoles/mg. of protein/hr.
(Whole dispersion)	2400	23·3	37·5	365	719	6·98	327	3·17
N	230	24·2	2·75	278	50	0·51	29	2·95
Mt_1	337	7·4	17·7	387	487	10·7	117	2·56
Mt_2	42	14·3	1·05	362	63	21·7	11	4·00
Ms	312	22·6	7·2	521	24	1·7	80	5·81
S	1337	40	7·8	237	0	0	71	1·77

min. (Table 1) were treated with a number of reagents with the intention of solubilizing the enzyme, and after treatment (Table 4) the suspensions were centrifuged at $6{\cdot}2 \times 10^6 g$-min. Without the added reagents, all activity was deposited, but a number of the reagents left measurable activity in the supernatant fraction. This occurred to a small extent only after treatment with organic solvents or bicarbonate (1, 5 and 6 of Table 4), but surface-active agents were more effective. Deoxycholate and also Lubrol W (a condensate of cetyl alcohol and a polyoxyethylene) gave supernatants of which the adenosine triphosphatase measured in the absence of sodium salts showed 30–40 % of its value in the original fraction Ms. Sodium activation, however, was almost completely lost in the supernatants. With deoxycholate the deposited material retained some degree of activation by sodium, though less than shown by the starting material.

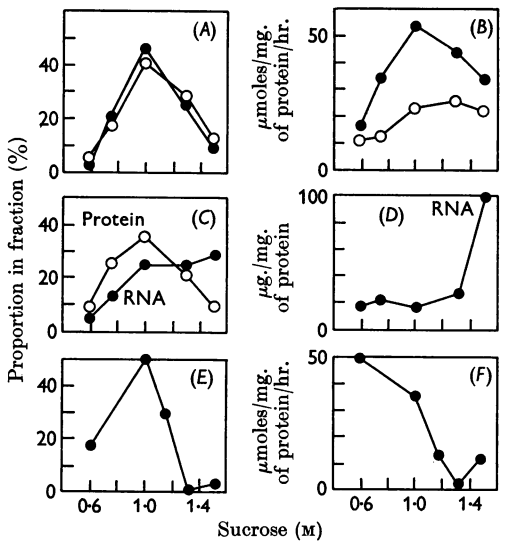

Fig. 5. Density-gradient separation of microsomal material from guinea-pig cerebral cortex. Right-hand curves (*B*, *D*, *F*) give the distribution of adenosine triphosphatase, ribonucleic acid and cholinesterase in terms of protein content; for assay conditions, see Experimental section. Left hand curves (*A*, *C*, *E*) express the distribution of a given component as a percentage of its total quantity or activity. Adenosine triphosphatase (*A* and *B*): ○, without sodium; ●, with 100 mM-Na.

Success in obtaining a soluble, sodium-activated enzyme was achieved with digitonin. This reagent was adopted in the knowledge that the microsomal material was rich in cholesterol (Table 3), and following the use of digitonin in solubilizing a muscle adenosine triphosphatase (Barbato & Alpert, 1961). With cerebral microsomal fractions (Table 4), digitonin was equally effective immediately after their preparation, and also after a preparation had been kept for 20 days at −20°.

Effects of added agents on the microsomal adenosine triphosphatase

In examining the actions of added substances, special attention has been given to any differential effects associated with the presence or absence of

Table 4. *Results of attempts to dissolve the microsomal adenosine triphosphatase*

Samples of fraction Ms (Table 1), 1–4 days after preparation from guinea-pig cerebral cortex, were treated in 0·32M-sucrose at pH 7·4 for 1 hr. at 0° unless otherwise stated, after which tubes were centrifuged at 104 000*g* for 60 min. In the solution and deposit protein was determined, and adenosine triphosphatase was assayed in standard reaction mixtures with and without 100 mM-Na.

	Adenosine triphosphatase (μmoles of phosphate/mg. of protein/hr.)					
	Original Ms		Deposit		Supernatant	
Treatment	Without Na	With Na	Without Na	With Na	Without Na	With Na
(1) 0·2M-$KHCO_3$, pH 8·8–9·3, 16 hr.	13	33	6·3	23	2·1	2·1
(2) Lubrol W, 0·1 %	28·2	49·5	13·7	13·4	19·1	20·1
(3) Deoxycholate, 0·1 %	28·2	49·5	19·3	26·5	10·3	11·5
	—	—	—	—	7·4*	8·1*
(4*a*) Digitonin, 0·5 %, 3 hr., 0·16M-sucrose	10·8†	21·6†	10·4	23·1	9·6	20·0
(4*b*) Digitonin, 0·5 %, 2·5 hr., 0·16M-sucrose	15·7†	33·2†	16·0	23·6	18·0	24·6
(5) Acetone-dried powder, suspended in water	26	50	—	—	1·0	0·7
(6) Slices‡ exposed to $CHCl_3$ at −20° to −40°, 3 days	28	42	—	—	2·8	2·6

* Centrifuging commenced immediately after mixing.

† Values quoted are for a control preparation treated with 0·16M-sucrose, centrifuged and the deposit assayed: (*a*) treated immediately after preparation; (*b*) a different batch treated 20 days after preparation.

‡ Values quoted are for microsomal material prepared as Ms from batches of the slices before and after treatment.

sodium salts. Ouabain shows specificity in this sense (Table 5), affecting preferentially the sodium-activated adenosine triphosphatase, as has already been shown in other systems.

On the other hand, the action of two strongly acidic substances proved to be independent of the presence of sodium: suramin, the trypanocidal agent bis-(*m*-amino-*m*-aminobenzoyl-*p*-methylbenzoyl-1-naphthylamine-4,6,8-trisulphonate)carbamide, and 2,4,6-trinitrobenzenesulphonate. The values of Table 5 show almost the same percentage inhibition by these agents in the presence and absence of sodium salts. The effect of suramin (see Thomson & McIlwain, 1961) was antagonized by protamine. 2,4-Dinitrophenol, 50 and 300 μM, was not found to affect the enzyme in the presence of sodium salts. Ganglioside preparations (0·15 and 0·3 mg./ml.) in the absence of sodium had little effect on the enzyme, increase or decrease of 12 % or less being observed. In the presence of sodium salts inhibition was found (Deul & McIlwain, 1961*a*, *b*); values are included in Fig. 3.

Protamine: chemical studies. Protamine and components of the assay mixture were observed to undergo chemical reaction independently of the tissue preparation. This was examined in detail (Table 6) as it involved the alkali metals, magnesium and adenosine triphosphate in a fashion which seemed relevant to adenosine-triphosphatase activity.

A solution made up to contain 2 μequiv. of protamine/ml. and 3 mM-tris–adenosine triphosphate, gave a precipitate at 0° or 37°. At 37°, the solution was centrifuged and the supernatant found by absorption at 260 mμ to be 2·2 mM in adenosine triphosphate; the Sakaguchi reaction showed protamine in the precipitate. Adenosine diphosphate gave no precipitate under these or the other conditions examined. The precipitate with adenosine triphosphate formed also in the presence of 100 mM-tris, of 3 mM-Mg or 30 mM-K, or in combinations of the reagents at these concentrations.

Increasing quantities of sodium or potassium, especially with 3 mM-Mg, were, however, effective in preventing the formation of the precipitate. At 100 mM-Na or -K, and 3 mM-Mg, precipitates were not formed at 37° but were formed at 0°. A precipitate already formed from protamine and adenosine triphosphate in a solution containing 3 mM-Mg, redissolved at 0° or at 37° when made

Table 5. *Substances modifying the microsomal adenosine-triphosphatase activity*

Microsomal fractions were prepared from guinea-pig cerebral cortex according to Table 1 and kept at −20° for the number of days stated below. The added agents were examined in standard reaction mixtures, which were mixed and left at 0° for 15 min. before being placed at 37° and before the reaction was started by addition of the adenosine triphosphate. Expts. 1 and 2: preparations kept 30–35 days (similar results were obtained with two other preparations). Expt. 3: preparation kept 20 days (similar inhibitions were shown with four other preparations kept for 11–180 days). Expts. 4, 6 and 7: preparations kept 26–30 days (a similar action of clupein was shown in three other preparations). Expt. 5: preparation kept 180 days.

Expt. no.	Added agent (μM, unless specified otherwise)	Adenosine triphosphatase (μmoles of phosphate/mg. of protein/hr.) Without Na	100 mM-Na
1	None	14·4	35·8
	Ouabain 0·1	15·1	29·1
	1	15·1	25·7
	10	15·1	21·2
	100	14·4	17·9
2	None	21·2	36·5
	Suramin 340	6·3	10·9
	Suramin 340 (with clupeine, 0·5 mg./ml.)	17·5	31·8
3	None	21·5	34·0
	2,4,6-Trinitrobenzenesulphonate 100	14·7	23·7
	200	11·0	17·2
4	None	8·6	25·4
	Clupeine 0·25 mg./ml.	13·8	19·9
	0·5 mg./ml.	11·3	17·9
5	None	12·5	22·9
	Clupeine 0·25 mg./ml.	24·5	22·0
	0·5 mg./ml.	18·6	22·0
6	None	14·4	28·8
	Salmine 0·5 mg./ml.	16·9	21·8
7	None	14·4	29·8
	Poly-L-lysine 0·5 mg./ml.	18·9	30·0

Table 6. *Precipitation of protamine adenosine triphosphate modified by sodium, potassium and magnesium*

Mixtures contained 2 μequiv. of protamine sulphate/ml., 3 mM-tris–adenosine triphosphate unless otherwise specified, and the chlorides listed below.

Further constituents of mixture (mM)				Precipitate	
Mg	K	Na	Other change	At 0°	At 37°
0	0	0	0	+	+
0	0	0	No tris	+	+
0	0	0	ADP for ATP	0	0
3	0	0	0	+	+
3	30	0	0	+	+
3	100	0	0	+	0
3	0	100	0	+	0
3	30	100	0	+	0
0	30	100	0	+	+
3*	30	100	0	+	0
3	100*	0	0	+	0
3	0	350*	0	0	0
12*	30	100	0	0	0
12	30	100	EDTA†, 100	+	+

* Reagent added last, after a precipitate had already formed.
† Reagent added after a precipitate had formed and redissolved.

350 mM in NaCl. The 3 mM-Mg also diminished the precipitate formed in the presence of smaller concentrations of sodium or potassium. Increase in magnesium to 12 mM did not dissolve an already formed precipitate, but it made 100 mM-Na more effective in doing so, for solution occurred at 0° with 12 mM-Mg and 100 mM-Na. Addition of excess of EDTA now caused the precipitate to reappear. Magnesium plus one of the alkali metals thus appears to be able to compete with protamine in salt formation with adenosine triphosphate, the protamine complexes being insoluble and the magnesium–sodium (or magnesium–potassium) complexes being soluble. In the standard reaction mixture of 3 mM-Mg, 100 mM-Na and 30 mM-K at 37°, the adenosine triphosphate appears likely to be present largely as a salt with magnesium and sodium.

Protamine and adenosine triphosphatase. In the standard-assay system with 100 mM-NaCl, protamine at approx. 1 and 2 μequiv./ml. remained completely in solution and inhibited the reaction, to an extent of between 5 and 30 %, when examined in five different microsomal preparations. These had been kept at −20° for 13–180 days, and the inhibition was not markedly dependent on whether the enzyme had received a prior exposure to protamine, of 15 min. at 0°, in the reaction mixture lacking only adenosine triphosphate. In view of the chemical interactions just described, inhibition by protamine was not surprising, but more unexpected was the effect of protamine in reaction mixtures lacking sodium. Here protamine accelerated the reaction, in each of six experiments with different enzyme preparations variously kept and mixed with the reagents; examples are quoted in Table 5. The acceleration caused by clupeine was between 30 and 100 % and could approach that caused by sodium; salmine and the poly-L-lysine also accelerated. In these reaction mixtures, clupeine at 0·5 mg. (2 μequiv.)/ml. formed the precipitate previously described, which removed some of the adenosine triphosphate and protamine from solution. At 1 μequiv./ml. little precipitate formed and, on each of three occasions on which it was examined, this concentration of protamine had the greater effect in stimulating the adenosine triphosphatase.

DISCUSSION

Localization and activity of the adenosine triphosphatase

Present findings confirm the association of the sodium-activated enzyme with microsomal material and show that of such material the association is with membrane structures and not with ribonucleic acid granules. The method of preparation adopted gave 75 % of the sodium-stimulated activity in a single fraction (Table 2), observed to liberate in the standard reaction mixture up to 62 μmoles of phosphate/mg. of protein/hr. (Fig. 3), or 855 μmoles of phosphate/hr. by microsomal material from 1 g. of tissue. The potency of the whole tissue so tested is, presumably, 855 × 4/3, or 1140 μmoles of phosphate/g. of tissue/hr. With increase in concentration of adenosine triphosphate this rate can probably be increased in the ratio of 110/62 (Fig. 4); it would then come close to the value of 1500 μmoles of phosphate/g./hr. estimated in the cerebral cortex of the cat by Bonting, Simon & Hawkins (1961), whose

assays depended on inhibition of the enzyme by ouabain and did not involve separation of subcellular fractions. Both these values are to be regarded at present as approximate only and dependent on conditions of tissue preparation and of assay, but they emphasize that at least half the total adenosine-triphosphatase activity of the tissue, of some 2000 μmoles of phosphate/g. of tissue/hr. (Gore, 1952), has the characteristics of the enzyme now being studied. This appraisal gives a higher activity/unit weight of tissue than found by Deul & McIlwain (1961*b*) and one much higher than that of Järnefelt (1961; and personal communication).

Sodium salts added to the present fraction under standard conditions of assay in reaction mixtures with magnesium and potassium, approximately doubled its activity; ratios of 1·8–3·4 were obtained under the various conditions described above. Activation by alkali metals, averaging threefold, can be obtained if sodium and potassium are added to media containing magnesium (Deul & McIlwain, 1961*b*), and this method of appraising activation was employed by Skou (1962), who found that activation up to eightfold was obtainable with the two metals. We have, however, preferred to base the present fractionations on activation by sodium only as other adenosine triphosphatases can be activated by magnesium and potassium (Kielley, 1961). The fraction found by Skou (1962) to be activated eightfold showed activity with sodium, potassium and magnesium of 1130 μmoles of phosphate liberated/mg. of nitrogen/hr.; assuming the nitrogen to be that of protein with 16% of nitrogen, the rate corresponds to 180 μmoles of phosphate/mg. of protein/hr. This value is 65% above the maximal rate computed for the present preparation (Fig. 4), but the yield of the most active fraction of Skou (1962), per unit weight of tissue, is not available.

Protamine and the specificity of sodium activation

Basic polypeptides constitute the one group of substances so far recognized as comparable with sodium in the limited sense that they increase the microsomal adenosine-triphosphatase activity when added in the presence of magnesium and potassium salts. In Skou's (1957, 1960, 1962) investigation of the enzyme from crab nerve and mammalian brain, potassium as activator in the presence of sodium and magnesium could be replaced by a number of univalent cations, but no agent was reported that replaced sodium in a system containing magnesium and potassium. In the present experiments, activation of the cerebral microsomal adenosine triphosphatase was shown by synthetic poly-L-lysine and by the mixture of arginine-rich peptides which constitute (Felix, 1960; Callanan *et al.* 1957) the protamines clupeine and salmine. Activation by these agents was shown in all the cerebral microsomal preparations examined; activity was usually increased to a smaller extent than by sodium itself, but required a very much smaller molar quantity of protamine than of sodium. Thus the half-maximal activation which was given by 15 mM-sodium was given by less than 1 μequiv. of protamine/ml., or probably about 0·1 mM.

The extent to which both protamine and sodium interact with adenosine triphosphate as well as with the triphosphatase merits emphasis here. Through the precipitate formed by protamine and adenosine triphosphate in the presence of the buffer and magnesium salts of the standard reaction mixture, and also the solution of the precipitate, it was concluded (see above) that protamine could compete with magnesium, potassium and sodium for salt formation with the triphosphate. The concentrations in which the components took part in these interactions were similar to those in which they modified the activity of the enzyme. Solution of the protamine adenosine triphosphate occurred equally well with either sodium or potassium and was facilitated by magnesium. The observations are sufficient to suggest that when protamine activates the enzyme, the substrate may be protamine ATP or protamine magnesium ATP, in place of (sodium and/or potassium) magnesium ATP as normal substrate; alternatively, some phenomenon akin to uncoupling may have occurred. The facility with which ATP forms magnesium–alkali-metal complexes has been emphasized by Lowenstein (1960), who showed that a non-enzymic transphosphorylation between ATP and inorganic phosphate was catalysed by magnesium plus alkali-metal salts.

Accepting the normal substrate of the enzyme system as (sodium and/or potassium) magnesium ATP, acceleration of the reaction by both of the alkali metals indicates the enzyme or associated structures to have specific affinities or conformations related to both sodium and potassium. Also, one interpretation of the Lineweaver–Burk plot of Fig. 4 is that, under the experimental conditions concerned, sodium increases the affinity of the enzyme for magnesium potassium ATP. Though other interpretations are possible, the extrapolation gives the concentration of ATP at which half-maximum velocity is attained as 20 mM in the absence and 8·7 mM in the presence of 100 mM-sodium, whereas the maximum velocity is the same with or without sodium. If the site with sodium affinity includes an acidic group it is understandable that it should combine also with protamine, for protamine forms complexes with many proteins and other substances or molecular aggre-

gates of large molecular weight, containing acidic groupings (see Sela & Katchalski, 1959; McIlwain, 1959, 1961).

Relationship to ion transport

Both adenosine triphosphate and the adenosine triphosphatase have thus displayed properties which are relevant to a role in ion transport, and which extend beyond the properties of adenosine triphosphate as an energy-rich substrate. In particular, both adenosine triphosphate and the adenosine triphosphatase of the microsomes show affinity for sodium. Present findings with substances that affect the triphosphatase suggest an involvement of acidic and basic centres in the enzyme action. Thus it was concluded by Kubo *et al.* (1960) that 2,4,6-trinitrobenzenesulphonate reacted with the ϵ-amino groups of two lysine residues which were involved in myosin adenosine triphosphatase. The enzyme was then inhibited by using the sulphonate at 30–330 μM and pH 7·5, and in the present study 100–200 μM inhibited at pH 7·4. The strongly acidic suramin was equally potent as an inhibitor, though it is more likely to act by salt formation than by the covalent linkage considered to be formed with trinitrobenzenesulphonate.

The property of the basic polypeptides most relevant to their activation of the enzyme appears likely to be the sequence of positive charges which has been invoked in interpreting other of their biological actions (Sela & Katchalski, 1959). Presumably such charges could assist the association of enzyme and substrate by combination with the acidic groups of each, but the basic polypeptides would be unable to undergo the subsequent movements that the enzyme initiates in the alkali metals. The polypeptides block active ion movements at cerebral tissues (McIlwain *et al.* 1961).

In considering how such movements are caused, present considerations emphasize the finding that both adenosine triphosphate and the adenosine triphosphatase of the microsomes show affinity for sodium, and that the enzyme has other affinities for the triphosphate implied by the hydrolysis it catalyses. Thus the coming together of enzyme and substrate itself implies an active movement of sodium. As the enzyme is attached to membrane structures, this involves movement of sodium in a particular direction in relation to the membrane. Such vector qualities in enzyme action (see Koshland, 1960) have been emphasized in this respect in a proposal (McIlwain, 1962) of how the present enzyme system may function.

SUMMARY

1. By differential centrifuging of sucrose dispersions of guinea-pig cerebral cortex, 75 % of their sodium-stimulated adenosine-triphosphatase activity was obtained in a microsomal fraction; the tissue activity was computed as 1100–1500 μmoles of phosphate formed/g. of fresh tissue/hr.

2. Assay conditions for the enzyme, and changes in its activity on keeping, are described. The optimum pH was 7·0–7·4; half-maximal activity with 3 mM-adenosine triphosphate, 3 mM-Mg and 30 mM-K was given by 15 mM-Na. With 3 mM-Mg, 30 mM-K and variable adenosine triphosphate, extrapolation suggested half-maximal activity at 20 mM-ATP; with 100 mM-Na in addition, the value became 8·7 mM-ATP.

3. Differential and density-gradient centrifuging indicated the enzyme system to be associated with the membrane structures rather than the ribonucleic acid granules of the microsomal fraction. Fractions richest in the adenosine triphosphatase possessed negligible succinic dehydrogenase but were enriched in acetylcholinesterase and in cholesterol.

4. A proportion of the adenosine-triphosphatase activity was no longer deposited at $6{\cdot}2 \times 10^6 g$-min. after treatment of microsomal fractions with deoxycholate, with Lubrol W or with digitonin; with the last-named reagent stimulation by sodium was retained to an appreciable degree by the enzyme of the supernatant.

5. The adenosine-triphosphatase activity of untreated microsomal fractions was inhibited by 2,4,6-trinitrobenzenesulphonate and by suramin, in reaction mixtures with magnesium and potassium, or with magnesium, potassium and sodium salts. Ouabain at 10^{-7}–10^{-4} M inhibited with magnesium, potassium and sodium but not without sodium. Basic polypeptides activated with magnesium and potassium but not with magnesium, potassium and sodium. Competition was noted between protamine, magnesium and alkali metals for salt formation with adenosine triphosphate.

6. The data are discussed in relation to the likely functioning of the enzyme system in active sodium and potassium transport.

We are grateful to Miss B. Aylett for assistance during this study and to those stated in the text as supplying substances or analytical values. It was carried out during the tenure by A.S. of Grant HPD 11,875-Cl from the United States Public Health Service, National Heart Institute, and by H.S.B. of a C. J. Martin Fellowship from the National Health and Medical Research Council of Australia. Preliminary studies were carried out by M. A. Spirtes and R. J. Woodman, whom we thank.

REFERENCES

Aldridge, W. N. (1962). *Biochem. J.* **83**, 527.

Aldridge, W. N. & Johnson, M. K. (1959). *Biochem. J.* **73**, 270.

Barbato, I. M. & Alpert, N. R. (1961). *Arch. Biochem. Biophys.* **93**, 255.
Bonting, S. L., Simon, L. A. & Hawkins, N. M. (1961). *Arch. Biochem. Biophys.* **95**, 416.
Booth, D. A. (1962). *J. Neurochem.* **9**, 265.
Callanan, M. J., Carroll, W. R. & Mitchell, E. R. (1957). *J. biol. Chem.* **229**, 279.
Carroll, W. R., Callanan, M. J. & Saroff, H. A. (1959). *J. biol. Chem.* **234**, 2314.
Deul, D. H. & McIlwain, H. (1961*a*). *Biochem. J.* **80**, 19P.
Deul, D. H. & McIlwain, H. (1961*b*). *J. Neurochem.* **8**, 246.
Emanuel, C. F. & Chaikoff, I. L. (1957). *Biochim. biophys. Acta*, **24**, 254.
Felix, K. (1960). *Advanc. Protein Chem.* **15**, 1.
Gibson, K. D., Wilson, J. D. & Udenfriend, S. (1961). *J. biol. Chem.* **236**, 673.
Gore, M. B. R. (1952). *Biochem. J.* **50**, 18.
Green, A. & Mommaerts, W. F. H. M. (1954). *J. biol. Chem.* **210**, 695.
Hanzon, V. & Toschi, G. (1959). *Exp. Cell Res.* **16**, 256.
Hanzon, V. & Toschi, G. (1960). *Exp. Cell Res.* **21**, 332.
Hele, P. & Finch, L. (1960). *Biochem. J.* **75**, 352.
Järnefelt, J. (1961). *Exp. Cell Res.* **25**, 211.
Kielley, W. W. (1961). In *The Enzymes*, vol. 5, p. 159. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Koshland, D. E. (1960). *Advanc. Enzymol.* **22**, 45.
Kubo, S., Tokura, S. & Tonomura, Y. (1960). *J. biol. Chem.* **235**, 2835.
Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.
Lowenstein, J. M. (1960). *Biochem. J.* **75**, 269.
Lowry, O. H., Roberts, N. R., Wu, M.-L., Hixon, W. S. & Crawford, E. J. (1954). *J. biol. Chem.* **207**, 19.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McIlwain, H. (1959). *Biochem. J.* **73**, 514.
McIlwain, H. (1961). *Biochem. J.* **78**, 24.
McIlwain, H. (1962). In *Symp.: Enzymes and Drug Action.* Ed. by Mongar, J. L. & de Reuck, A.U.S. London: J. and A. Churchill.
McIlwain, H. & Rodnight, R. (1962). *Practical Neurochemistry.* London: J. and A. Churchill.
McIlwain, H., Woodman, R. J. & Cummins, J. T. (1961). *Biochem. J.* **81**, 79.
Perry, S. V. & Chappell, J. B. (1957). *Biochem. J.* **65**, 469.
Quastel, J. H. & Wheatley, A. H. M. (1938). *Biochem. J.* **32**, 936.
Schwartz, A. (1962). *Biochem. Pharmacol.* **11**, 389.
Sela, M. & Katchalski, E. (1959). *Advanc. Protein Chem.* **14**, 391.
Showacre, J. L. & Du Buy, H. G. (1955). *J. nat. Cancer Inst.* **16**, 173.
Skou, J. C. (1957). *Biochim. biophys. Acta*, **23**, 394.
Skou, J. C. (1960). *Biochim. biophys. Acta*, **42**, 6.
Skou, J. C. (1962). *Biochim. biophys. Acta* (in the Press).
Thomson, C. G. & McIlwain, H. (1961). *Biochem. J.* **79**, 342.
Toschi, G. (1959). *Exp. Cell Res.* **16**, 232.
Wherrett, J. & McIlwain, H. (1962). *Biochem. J.* **84**, 232.
Whittam, R. (1961). *Nature, Lond.*, **191**, 603.

Biochem. J. (1962) **84**, 637

Quinones and Related Compounds in Fish Tissues

BY J. F. PENNOCK, R. A. MORTON, D. E. M. LAWSON* AND D. L. LAIDMAN†
Department of Biochemistry, University of Liverpool

(*Received* 5 *April* 1962)

At least five ubiquinones occur naturally (Lester & Crane, 1959). They differ only in the number of isoprene residues in a side chain of 30, 35, 40, 45 or 50 carbon atoms. The lower members are in the main characteristic of micro-organisms and the higher members, principally ubiquinone-50, are found in animals.

In fishes two forms of vitamin A occur; vitamin A_2 (a dehydrovitamin A_1) preponderates in most freshwater species, but vitamin A_1 preponderates in marine species (for references see Morton, 1960). This phenomenon is somewhat isolated and it is of interest to inquire whether it has a parallel in any new family of biologically active compounds. With this aim before us we have examined the ubiquinones in the hearts from various species of freshwater and marine fishes as well as two anadromous species. The occurrence of ubiquinone in fishes is to be expected but few observations have previously been recorded (see Lester & Crane, 1959; Pennock, Morton & Lawson, 1959).

During this work the amounts of several minor constituents of the unsaponifiable matter from fish tissues were also determined.

* Holder of a Research Studentship from the Department of Scientific and Industrial Research.
† In receipt of a Research Studentship from the Agricultural Research Council.

MATERIALS AND METHODS

Fish tissues. Hearts, livers, and white flesh of the cod *Gadus callarias* were obtained from the Torry Research Station, Aberdeen, by the courtesy of the director Dr G. A.