Separation of Adenosine Diphosphate-Adenosine Triphosphate-Exchange Activity from the Cerebral Microsomal Sodium-plus-Potassium Ion-Stimulated Adenosine Triphosphatase

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1. A microsomal fraction from ox cerebral cortex catalysed [^{14}C]ADP-ATP exchange at a speed similar to that at which it liberated P_i from ATP in the presence of Na^+ , K^+ and Mg^{2+} . 2. Repeated washing the fraction with MgATP solutions solubilized most of the exchange activity and left the adenosine triphosphatase insoluble and little changed in activity. The exchange activity was accompanied by negligible adenosine-triphosphatase activity and was enriched by precipitation at chosen pH and by DEAE-Sephadex. At no stage was its activity affected by Na^+ , K^+ or ouabain. 3. The washed microsomal fraction was exposed to a variety of reagents; a sodium iodide-cysteine treatment increased both adenosine-triphosphatase and exchange activities, as also did a synthetic zeolite. Preparations were obtained with exchange activities less than 3% of their Na^+ -plus- K^+ -stimulated adenosine-triphosphatase activity. Some contribution to the residual exchange activity was made by an adenylate kinase. 4. Thus over 95% of the microsomal ADP-ATP-exchange activity does not take part in the Na^+ -plus- K^+ -stimulated adenosine-triphosphatase reaction. Participation of some of the residual 3% of the ADP-ATP-exchange activity has not been excluded, but there appears no firm evidence for its participation in the adenosine triphosphatase; the bearing of this conclusion on mechanisms proposed for the Na^+ -plus- K^+ -stimulated adenosine triphosphatase is indicated.

Mechanisms for the utilization of ATP in active movements of Na^+ and K^+ , referred to in the preceding paper (Swanson & Stahl, 1966), are at present in the stage of formulation. As a result of his study of a crab-nerve preparation, Skou (1957, 1960) concluded that a critical role was played by a Na^+ -plus- K^+ -activated ATPase† that had also the property of catalysing the exchange of [^{14}C]ADP with ATP. This exchange reaction was regarded as a property of a single ATPase and was considered to demonstrate an 'energy-rich' phosphate as intermediate in the ATPase (see the Discussion section). Preparations catalysing both the exchange and the ATPase reactions have been made also from

Electrophorus electricus and a similarity in the rates of the two reactions has been reported (Albers, Fahn & Koval, 1963); also, kidney preparations catalysed the two reactions (Post, Sen & Rosenthal, 1965). Here again the exchange was regarded as due to a single enzyme, and to be a partial reaction of the (Na^+ , K^+)-ATPase. These assumptions were incorporated in an extensive theoretical treatment of the reaction mechanism of the ATPase (Post & Sen, 1965).

To study these enzymes more objectively it is desirable to release them from the microsomal structure and to attempt their purification. The ATPase could be readily obtained in the form of a micellar solution by surface-active agents (Swanson, Bradford & McIlwain, 1964), but separation of enzyme activity from other membrane components was not easily achieved (Swanson & Stahl, 1966). However, we have found (Stahl, Sattin & McIlwain, 1965) that the bulk of the ADP-ATP-exchange activity may be recovered from cerebral microsomes in soluble form by using a simple extraction

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‡ Abbreviations: ATPase, adenosine triphosphatase; (Na^+ , K^+)-ATPase, Na^+ -plus- K^+ -stimulated adenosine triphosphatase.

procedure. The (Na⁺,K⁺)-ATPase remained in particulate form and was essentially unaffected by this procedure. With further treatment it has been possible to achieve still greater reduction of exchange activity in microsomes so that only 4% of that originally present remains. This prompts a re-evaluation of reaction mechanisms in which these enzymes have been involved, as is indicated in the Discussion section.

EXPERIMENTAL

Cerebral preparations

Ox brains were removed at the slaughter-house within 15–20 min. after the death of the animals, and were immediately placed in a water-tight polythene bag in crushed ice. The average lapse of time between death of the animals and arrival of specimens at the laboratory was 1.5 hr.

Preparation of subcellular fractions. In the cold room (4–5°), grey matter was snipped from the cerebral cortices with scissors, after removal of meninges, yielding nearly 35 g. of tissue/brain. This was homogenized in a top-drive macerator (Mark II; Townson and Mercer Ltd., Croydon, Surrey) for 1 min. with 9 ml. of 0.32 M-sucrose, pH 7.4 (adjusted with dilute KOH)/g. wet wt. of tissue. All subsequent operations were carried out at 0–7°.

The tissue suspension was centrifuged at 6500 g for 15 min. in an MSE High-Speed 17 refrigerated centrifuge (angle rotor 69179) to remove material including erythrocytes and other unbroken cells, nuclei, mitochondria and nerve endings. The residue was resuspended in the top-drive macerator for 15 sec. with 9 ml. of 0.32 M-sucrose, pH 7.4/g. wet wt. of original grey matter, and was centrifuged at 6500 g for 15 min. Supernatants from these operations were combined and re-centrifuged at 10400 g for 15 min.; all the residues (designated N + Mt₁) were combined, and the supernatants centrifuged at 23500 g for 1 hr. (angle rotor 69180 of MSE centrifuge or no. 21 angle rotor of Spinco model L ultracentrifuge) to yield a supernatant and pellet. This pellet was homogenized in a Teflon-and-glass homogenizer (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) with 1 ml. of 0.32 M-sucrose, pH 7.4/g. wet wt. of original tissue, and was centrifuged at 9800 g for 15 min. The resulting pellet and supernatant fractions were designated Mt₂ and microsomes respectively.

Preparation of microsomal extract. One-half volume of a solution 1 mM in Na₂ATP and 1 mM in MgCl₂, adjusted to pH 7.4 with KOH, was mixed with the suspension of microsomes. After a period of 5 min. or longer the mixture was centrifuged at 78000 g for 1 hr. in the no. 30 rotor of the Spinco model L preparative ultracentrifuge. The supernatant was saved, and the pellet was resuspended in 1.5 ml. of the ATP-MgCl₂ solution/ml. of original microsomal suspension, by five or six passes of the pestle in a Teflon-and-glass homogenizer. After centrifugation, as before but for 0.5 hr., the washing procedure was repeated and the three supernatants were combined to yield the MgATP microsomal extract. The residual pellet was designated 'washed microsomes'.

All supernatants were combined and dialysed for 4 hr. against distilled water at 5°.

Treatment of microsomes with synthetic zeolite. A micro-

somal pellet after the MgATP washing was transferred to a stainless-steel centrifuge tube cooled in ice, and molecular-sieve powder (a synthetic zeolite) was gradually added (3 g. of zeolite/g. wet wt. of pellet), giving a paste that was continuously mixed with a large glass rod for 5–10 min. The mixture was then extracted by mixing gradually with 15 ml. of ice-cold glass-distilled water/g. wet wt. of original pellet and centrifuging at a low speed at 0° to remove the zeolite. The supernatant was decanted, and the tightly packed pellet was extracted again with cold water as before and re-centrifuged. The combined supernatants were re-centrifuged to remove residual zeolite and the final supernatant was centrifuged at 105000 g for 1 hr. to yield a 105000 g supernatant (Expt. 3, e in Table 3) and 105000 g pellet (Expt. 3, d in Table 3).

Treatment of microsomes with sodium iodide. An MgATP-washed microsomal pellet was suspended in ice-cold glass-distilled water at a final concentration of 3 mg. of protein/ml. Then 1 vol. of the solution described by Nakao, Tashima, Nagano & Nakao (1965) was slowly added at 3°. The final concentrations were: NaI (1.97 M), Na₂EDTA (2.5 mM), MgCl₂ (2.5 mM), Na₂ATP (1.5 mM) and cysteine (25 mM), the whole being adjusted to pH 8.0 with tris. The suspension after 0.5 hr. at 3° was diluted with ice-cold glass-distilled water to yield a final concentration of 0.8 M-NaI and was centrifuged at 20000 g for 0.5–2 hr. to yield a pellet and a supernatant. The pellet was washed twice with 5 mM-Na₂EDTA, pH 7.4, after suspension with a Teflon-and-glass homogenizer, and was centrifuged at 20000 g for 0.5 hr. to yield the final residue and supernatant.

All supernatants were combined and dialysed for 4 hr. against distilled water at 5°.

Chemicals

Unlabelled nucleotides were from the Sigma Chemical Co., St Louis, Mo., U.S.A.; [8-¹⁴C]ADP (34 mc/m-mole) was from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.; [8-¹⁴C]AMP (11.8 mc/m-mole), [8-¹⁴C]ATP (4.8 mc/m-mole) and carrier-free [³²P]P_i were from The Radiochemical Centre, Amersham, Bucks. Stock nucleotide solutions were prepared as the tris salts according to the method of Schwartz, Bachelard & Mollwain (1962). Crude hog pancreatic lipase was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., and bovine pancreatic ribonuclease (five-times crystallized) was from Mann Research Laboratories Inc., New York, N.Y., U.S.A. Molecular-sieve powder, type 3A (a synthetic zeolite; Linde Air Products Co., New York, N.Y., U.S.A.) was supplied by British Drug Houses Ltd., Poole, Dorset.

Assays and analyses

Adenosine triphosphatase. The procedure described by Swanson *et al.* (1964) was used for determinations in the presence and absence of Na⁺ and K⁺. Comparable values for activity in the absence of Na⁺ and K⁺ were obtained as part of the assay of ADP-ATP-exchange activity (see Swanson & Stahl, 1966).

[¹⁴C]ADP-ATP-exchange activity. The assay described by Swanson & Stahl (1966) was utilized. [¹⁴C]ADP, obtained as the tritium salt in 50% (v/v) ethanol, was evaporated to dryness in a stream of N₂ at 40–50°, and sufficient carrier

tris-ADP, pH 7.4, was added to adjust the specific activity to approx. 16000 counts/min./ μ mole of ADP.

Adenylate-kinase activity. The rate of this reaction was determined, commencing with [14 C]AMP, by the method described by Swanson & Stahl (1966). Sufficient carrier tris-AMP, pH 7.4, was added to adjust the specific activity to approx. 16000 counts/min./ μ mole of AMP.

[32 P] P_i -ATP exchange activity. A solution of 25 mM- KH_2PO_4 was adjusted to pH 7.4 with 0.1 N-KOH and a solution of carrier-free $H_3^{32}PO_4$ added to give 2.5×10^5 – 5×10^5 counts/min. in the 0.1 ml. that was added to each reaction mixture of final volume 0.5 ml. These mixtures also contained tris-ATP (3 mM), tris-ADP (3 mM), $MgCl_2$ (3 mM) and tris-HCl buffer, pH 7.4 (30 mM). The final phosphate concentration was 5 mM and the reaction was run and terminated as described for the ADP-ATP-exchange reaction. However, electrophoresis was carried out at pH 3.8 for 6 hr. and 350 v to allow complete separation of [32 P] P_i and ATP on the electrophoresis strips (cf. Sato, Thomson & Danforth, 1963).

Protein. For suspensions containing more than 1–2 mg. of protein/ml. sufficient 5% (w/v) sodium deoxycholate was added to clarify the suspensions, and then the biuret method (Gornall, Bardawill & David, 1949) was used. Protein in more dilute solutions was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). In both cases crystalline bovine plasma albumin was used as standard and good agreement between the two methods was found.

Inorganic phosphate. This was determined by the method of Martin & Doty (1949) as stated by Swanson *et al.* (1964).

RESULTS

Extraction of ADP-ATP-exchange activity. Preliminary experiments indicated that much ADP-ATP-exchange activity could be extracted from cerebral microsomal fractions by several aqueous solutions. These included potassium chloride (0.15 M), tris-hydrochloric acid buffer, pH 7.4 or 8.0 (0.05 or 0.10 M), and Na_2ATP , pH 7.4 or 8.0 (1–2 mM). However, the highest yield of activity in soluble form was obtained with a solution 1 mM in magnesium chloride and in Na_2ATP , pH 7.4. As shown in Fig. 1, after three or four extractions the washed microsomes retained only about 13% of their original ADP-ATP-exchange activity. The specific activity of the (Na^+ , K^+)-ATPase was little affected by this extraction procedure.

The ox-brain microsomal preparation used as starting material showed (Table 1) an ATPase activity per unit of protein, and also a response to Na^+ , which were similar to those of corresponding guinea-pig preparations (Deul & McIlwain, 1961; Schwartz *et al.* 1962). As also shown in Table 1, 42% of the ADP-ATP-exchange activity of the original microsomes was recovered in the extract and this extract carried negligible ATPase activity. The washed microsomes still possessed 78% of the original ATPase activity with only 13% of the initial exchange activity. Overall recovery of ATPase, exchange activity and protein were 79, 55

Fig. 1. (Na^+ , K^+)-ATPase (●) and [14 C]ADP-ATP-exchange (○) activities of cerebral microsomes after extraction with mM- Na_2ATP -mM- $MgCl_2$, pH 7.4, as indicated in the text. The results are mean values from two experiments. The ADP-ATP reaction mixture was of volume 0.5 ml. and contained: $MgCl_2$ (3 mM), tris-HCl buffer, pH 7.4 (30 mM), tris-ATP (3 mM) and tris-ADP (3 mM, 25000 counts/min.). The reaction was begun by addition of the microsomal fraction (approx. 0.1 mg. of protein) and after 10 min. was terminated by addition of 0.1 ml. of 10% (v/v) conc. HCl in methanol. The (Na^+ , K^+)-ATPase reaction was assayed independently in a volume of 1 ml. containing $MgCl_2$ (3 mM), tris-HCl buffer, pH 7.4 (30 mM), tris-ATP (3 mM), KCl (30 mM) and NaCl (100 mM). The reaction was begun by the addition of microsomal fraction (approx. 0.1 mg. of protein) and after 5 min. was terminated by the addition of 2 ml. of 10% (w/v) trichloroacetic acid. The P_i and adenine nucleotides were analysed as described by Swanson & Stahl (1966).

and 97% respectively. This loss of exchange activity is partly explained by a subsequent finding of a pH optimum of 8.3 for the partially purified exchange activity (see below). The results reported were obtained at pH 7.4, which is optimum for the exchange activity measured before extraction. Some advantage accrued from first preparing acetone-dried powders from microsomes; these afforded an average of 63% of the exchange activity in soluble form (again measured at pH 7.4), with less than 1% of the original ATPase activity. The washed acetone-dried powder still retained 86% of the (Na^+ , K^+)-ATPase activity and only 12% of the original exchange activity.

Partial purification and properties. The exchange activity of the $MgATP$ microsomal extract was purified about tenfold with 67% recovery (Table 2). Several initial attempts to fractionate with ammonium sulphate were carried out at various pH values, but these led to low recoveries of exchange activity. Enrichment was obtained with good recovery in the supernatant from precipitation at pH 5. The

Table 1. Solubilization of the ADP-ATP-exchange activity of cerebral microsomal preparations

Results are mean values from four (section A) or two (section B) preparations, each from 70g. of fresh ox cerebral cortex. Each preparation was washed three times with mm-Na₂ATP-mm-MgCl₂, pH 7.4, as described in the Experimental section, to yield the washed microsomes and the supernatant.

Material	Protein (mg.)	ATPase activity				[¹⁴ C]ADP-ATP-exchange activity		
		(μmoles of P _i formed/mg. of protein/hr.)		Na ⁺ -stimulated activity		(μmoles of [¹⁴ C]ADP exchanged/mg. of protein/hr.)	Total (μmoles of [¹⁴ C]ADP exchanged/fraction/hr.)	Distribution (%)
		Without Na ⁺	With Na ⁺	Total (μmoles of P _i formed/fraction/hr.)	Distribution (%)			
A								
Original microsomes	842	9.8	24.9	12720	100	20.6	17330	100
Washed microsomes	708	6.9	21.0	9980	78.4	2.6	2190	12.7
MgATP microsomal extract	112	0.5	1.0	56	0.4	86.1	7240	41.8
B								
Microsomal acetone- dried powder	392	12.5	32.6	7880	100	37.9	14860	100
Washed residue	330	10.5	30.9	6740	85.6	5.4	1718	11.6
MgATP microsomal extract	73	2.6	3.5	66	0.8	128	9344	62.9

freeze-dried pH 5 supernatant was in the first instance chromatographed on a column (26cm. × 2cm.) of DEAE-Sephadex A-50 (medium grade) at pH 7.0 with 50mm-potassium phosphate and increasing concentrations of sodium chloride up to 1M. Nearly all the exchange activity was recovered, with further enrichment, in the void volume of the column (i.e. it was not held by the column). Therefore batch treatment of the freeze-dried pH 5 supernatant was tried with coarse-grade DEAE-Sephadex A-50, and this gave nearly identical purification, which is reported in Table 2.

The presence of Na⁺, K⁺ or ouabain did not appreciably affect the ADP-ATP-exchange activity of the original microsomes, the washed microsomes, or of the DEAE-Sephadex filtrate. These experiments were carried out in the assay mixture for ADP-ATP exchange described in the Experimental section, with the microsomal preparations described in Table 2 and the extract described in Table 3. Ouabain (0.1 mM), or sodium chloride (25 or 100 mM), with or without potassium chloride (30 mM), caused less than 8% change in the exchange activity in the original microsomal fraction which was at 14.5 μmoles/mg. of protein/hr., in that of the washed microsomes, originally 5.3 μmoles/mg. of protein/hr., and in that of the DEAE-Sephadex filtrate, originally 642 μmoles/mg. of protein/hr.

Residual exchange activity of washed microsomal fractions. Prolonged extraction procedures with mm-Na₂ATP-mm-magnesium chloride, pH 7.4 or 8.0, 30mm-tris-hydrochloric acid buffer, pH 7.4, or potassium chloride (0.15M) were not effective in removing a significant proportion of the residual exchange activity of the washed preparation. To further evaluate the degree of association of the exchange activity with microsomal structure the preparation was treated by ultrasonic irradiation and enzymic digestion (Table 3).

After an initial ultrasonic treatment of 10 min. (Expt. 1A) 13% of the original microsomal ADP-ATP-exchange activity was still retained in particulate form although 52% of the original protein was solubilized. By first washing the microsomal preparation (Expt. 1B) with mm-Na₂ATP-mm-magnesium chloride, pH 7.4, 12% of the protein and 71% of the exchange activity could be recovered in soluble form, but 9% of the exchange activity still remained particulate. After treatment by ultrasonic irradiation for 5 min. only 6% of the exchange activity remained insoluble. However, an exact quantitative interpretation of these results is difficult since the overall exchange activities were greatly stimulated in Expts. 1A and 1B by the ultrasonic treatment. The total recovery of exchange activity was 196 and 138% respectively in these experiments. The ATPase activity was greatly diminished by the treatments and could not

Table 2. *Partial purification of soluble ADP-ATP-exchange enzyme(s)*

The MgATP microsomal extract was obtained from the microsomes derived from 70g. wet wt. of ox cerebral cortex, and was treated as follows. (a) pH5 treatment. To the MgATP microsomal extract, magnetically stirred in an ice bath, a solution 0.1 M in Na₂ATP and in MgCl₂ was slowly added to lower the pH to 5. After 5 min. mixing the cloudy suspension was deposited by centrifugation at low speed. The pH5 pellet was resuspended in cold distilled water or in 5–10 mM-tris-HCl buffer, pH 7.4, for assay. Enough N-NaOH was added to the pH5 supernatant to raise the pH to 7.4 and the neutralized supernatant was immediately freeze-dried. The neutralized freeze-dried pH5 supernatant could be stored at –20° for several months without appreciable loss of activity. (b) Batch treatment with DEAE-Sephadex. DEAE-Sephadex A-50 (coarse grade) was washed free from fines and equilibrated for several days with 20 mM-potassium phosphate buffer, pH 7.0. Then 30 ml. of a slurry of this DEAE-Sephadex in phosphate buffer containing the equivalent of 2g. of dry Sephadex was transferred to a small beaker. The neutralized freeze-dried pH5 supernatant (approx. 40mg. of protein) was dissolved in 10–15 ml. of cold glass-distilled water and was added to the DEAE-Sephadex. This mixture was magnetically stirred for 1 hr. and was filtered (requiring 10–15 min.) through a sintered-glass funnel. The DEAE-Sephadex filtrate was then concentrated overnight by ultrafiltration-dialysis against 20 mM-potassium phosphate buffer, pH 7.0. All operations were carried out at 5°.

Fraction	Protein (mg.)	Specific activity (μ moles of [¹⁴ C]ADP exchanged/mg. of protein/hr.)	Total activity (μ moles of [¹⁴ C]ADP exchanged/fraction/hr.)
MgATP microsomal extract	133	79.7	10 600
pH5 pellet (neutralized)	79	11.1	876
pH5 supernatant (neutralized)	41	236	9 680
pH5 supernatant (neutralized and freeze-dried)	40	202	8 080
DEAE-Sephadex filtrate	9.2	770	7 080

be regained by simple combination of 'ultrasonic pellet' and 'ultrasonic supernatant' fractions in various proportions.

Extracting an acetone-dried powder derived from cerebral microsomes with mM-Na₂ATP-mM-magnesium chloride, pH 7.4, solubilized nearly 24% of the original protein and 69% of the ADP-ATP-exchange activity (Expt. 2 in Table 3). Digestion of the washed acetone-dried powder with a crude pancreatic lipase preparation (having both lipase and protease activities) liberated a further 25% of protein and a further 4% of exchange activity. After these operations the particulate material still retained 2.5% of the original exchange activity but only 15% of the overall (Na⁺,K⁺)-ATPase activity.

When washed microsomes were treated with a zeolite (Expt. 3 in Table 3) by using a procedure similar to that described by Person & Zipper (1964), 43% of the protein was solubilized. This supernatant (Expt. 3, *e* in Table 3) fraction contained 580% of the exchange activity present in the washed microsomes (Expt. 3, *b* in Table 3) and 6% of the (Na⁺,K⁺)-ATPase activity. The pellet (Expt. 3, *d* in Table 3) retained 54% of the (Na⁺,K⁺)-ATPase activity and 58% of the ADP-ATP-exchange activity present in the washed microsomes (Expt. 3, *b* in Table 3). This great increase in activity of the ADP-ATP exchange on solubilizing by the zeolite parallels the increased activity after ultrasonic irradiation and after the treatment with detergents reported by Swanson & Stahl (1966).

The treatment with a sodium iodide-cysteine reagent (Expt. 4 in Table 3) followed a procedure applied by Nakao *et al.* (1965) to different starting materials. The treated ATPase, like that of Nakao *et al.* (1965), was now almost entirely dependent on added Na⁺ and was, moreover, of twice its original activity. Concomitantly, the specific activity of the ADP-ATP exchange (still in particulate form) was increased 2.6-fold and the total recovery of exchange activity increased 1.8-fold. The ADP-ATP exchange in the sodium iodide-treated preparation proceeded at 11.4% of the rate of the (Na⁺,K⁺)-ATPase, which is about the ratio of activities shown in the washed microsomes.

Crystalline ribonuclease, incubated with the washed microsomal preparation for 10 min. or more, decreased by 68% the residual ADP-ATP-exchange activity (Fig. 2; see also comment on Table 4 below). Some loss of ATPase activity also occurred. As a portion of the residual exchange activity persisted after several treatments, its characterization was attempted by the methods of the following section.

Contribution of known ADP-ATP-exchange reactions to the residual ADP-ATP exchange of microsomes. As shown for the guinea-pig microsomal preparation (Swanson & Stahl, 1966) significant amounts of adenylate-kinase activity contributed to the ADP-ATP exchange. In the original ox-brain microsomal preparation this contribution amounted to 42% of the rate of the ADP-ATP exchange (Table 4), and after three or four washings

Table 3. *Adenosine-triphosphatase and ADP-ATP-exchange activities of microsomal fractions after various treatments*

Expt. 1A. Microsomes (in 0.32M-sucrose, pH7.4) were suspended in an ice-cold solution of reduced glutathione (15mm), tris-ATP (4mm) and MgCl₂ (2mm) adjusted to pH7.4 with tris; the final protein concentration was 10.5mg./ml. and the final volume was 6.3ml. The suspension in a vessel (2.5cm. diam.) was immersed in ice and treated with ultrasonics for a total of 10 min. with 30 sec. bursts and 2-3 min. cooling periods, by using an MSE ultrasonic disintegrator fitted with a titanium probe (19mm. diam.) and operated at 18-20kvc./sec. The suspension was diluted to 12ml. with mM-Na₂ATP-mM-MgCl₂, pH7.4, and was centrifuged at 85000g for 2 hr. to yield an 'ultrasonic supernatant' and an 'ultrasonic pellet', which was suspended in ice-cold distilled water for assay. Expt. 1B. Microsomes were washed with mM-Na₂ATP-mM-MgCl₂, pH7.4, by using the procedure described in the text. The washed microsomal pellet was resuspended in the solution of Expt. 1A at 11.3mg. of protein/ml.; the final volume was 6.3ml. Treatment with ultrasonics was carried out for 5 min. and centrifugation as in Expt. 1A. Expt. 2. An acetone-dried powder of microsomes was prepared as described in the text and was washed with mM-Na₂ATP-mM-MgCl₂, pH7.4, by centrifuging. The washed pellet was resuspended in cold medium containing Na₂ATP (3mm), MgCl₂ (3mm), potassium pyrophosphate, pH8.2 (100mm), and crude pancreatic lipase powder (2.3mg.) in a final volume of 11 ml. with 4.1 mg. of protein/ml.; the final pH was 8.2. The suspension was mixed continuously in an ice bath for 1-25 hr. and was centrifuged at 85000g for 0.5 hr. The resulting pellet was homogenized in 1 ml. of ice-cold 0.15M-KCl and was recentrifuged as before. Supernatants from these operations were combined ('digest supernatant') and the final pellet was resuspended in cold distilled water for assay. Expt. 3. Microsomes were washed with mM-Na₂ATP-mM-MgCl₂, pH7.4, and were treated with zeolite by using the procedure described in the text. Expt. 4. Microsomes were washed with mM-Na₂ATP-mM-MgCl₂, pH7.4, and were treated with an NaI reagent by using the procedure described in the text.

Expt. no.	Fraction	Protein (mg.)	ATPase activity				[¹⁴ C]ADP-ATP-exchange activity		
			(μmoles of P _i formed/mg. of protein/hr.)		(μmoles of [¹⁴ C]ADP exchanged/mg. of protein/hr.)	(μmoles of [¹⁴ C]ADP exchanged/fraction/hr.)	Total activity		
			(μmoles of P _i formed/fraction/hr.)						
			Without Na ⁺	With Na ⁺					
1A	Microsomes	66	12.2	29.4	1135	19.9	1303		
	'Ultrasonic pellet'	26	2.5	10.4	205	6.4	169		
	'Ultrasonic supernatant'	34	0.5	1.3	27	69.3	2380		
	Microsomes	194	12.2	29.4	3330	19.9	3860		
1B	Washed microsomes	139	7.9	25.4	2430	2.6	362		
	MgATP microsomal extract	23	3.9	3.0	0	103	2730		
	'Ultrasonic pellet'	93	3.7	18.5	1380	2.5	232		
	'Ultrasonic supernatant'	56	0.4	2.5	118	42	2345		
2	Microsomal acetone-dried powder	102	10.7	36.5	2630	45.8	4670		
	Washed acetone-dried powder	63	10.1	37.9	1750	6.7	422		
	MgATP microsomal extract	24	0	0	0	134	3220		
	'Digest pellet'	25	2.0	17.4	385	4.6	115		
3	'Digest supernatant'	28	0	0	0	5.8	168		
	(a) Original microsomes	1797	10.0	24.2	25520	21.2	38180		
	(b) Washed microsomes	1387	6.5	19.1	18570	4.0	5550		
	(c) MgATP microsomal extract	288	0	0	0	82.0	23720		
4	(d) 105000g pellet from zeolite-treated (b)	638	3.02	18.7	10000	5.1	3250		
	(e) 105000g supernatant	596	0.47	2.3	1074	54.2	32250		
	Washed microsomes	270	8.6	27.5	5100	3.03	818		
	Residue after NaI reagent	156	2.1	68.4	10300	7.76	1212		
	Supernatant	22	3.9	8.6	103	10.8	237		

with $\text{mm-Na}_2\text{ATP}$ – $\text{mm-magnesium chloride}$, $\text{pH } 7.4$, the contribution was still 29%. These preparations were all free from $[\text{P}^{32}]\text{P}_i$ –ATP-exchange activity.

The presence of hexokinase, phosphofructokinase or creatine-phosphokinase reactions might give rise to exchange of labelled ADP into ATP under the conditions of the assay. However, the addition of glucose (10mm), glucose 6-phosphate (10mm), fructose 1,6-diphosphate (8.3mm) or creatine (10mm) did not affect the rate of the exchange reaction in the washed ox-brain microsomes.

Fig. 2. $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ (●), $[\text{C}^{14}]\text{ADP-ATP-exchange}$ (○) and adenylate-kinase (▲) activities of washed cerebral microsomes after digestion with ribonuclease. The results are mean values from two experiments. The incubation was carried out at 25° in an open shaking bath. The final volumes were 3 or 10 ml. and contained microsomes (7.5 mg. of protein/ml.), crystalline ribonuclease (0.5 mg./ml.), tri-ATP (3 mm), MgCl_2 (3 mm) and glycylglycine buffer, $\text{pH } 7.7$ (20 mm). At indicated intervals 1 ml. or 3 ml. samples were withdrawn and pipetted into 10 vol. of $\text{mm-Na}_2\text{ATP}$ – mm-MgCl_2 , $\text{pH } 7.4$, in tubes that were then centrifuged at 85000g for 2 hr. The resulting pellets were resuspended in cold glass-distilled water and assayed as described in the Experimental section.

Phosphoglycerate kinase is very active in cerebral tissues (Lowry & Passonneau, 1964) and operates at very low substrate concentrations. However, the addition of 3-phosphoglycerate (0.16 μM) caused no change in the rate of the ADP–ATP exchange. The presence of pyruvate (10 mm) also did not affect the exchange. This would appear to exclude the presence of significant amounts of enzymes transferring phosphate to these substrates.

Succinyl-CoA synthetase causes incorporation of labelled ADP into ATP (Kaufman, 1955). Additions of succinate and CoA are necessary for exchange of $[\text{P}^{32}]\text{P}_i$ with ATP in the purified enzyme. However, additions of succinate (50 or 100 mm) plus CoA (0.47 mm) with or without reduced glutathione (16 mm) did not affect the ADP–ATP exchange and did not elicit a $[\text{P}^{32}]\text{P}_i$ –ATP exchange in the washed microsomes, thus excluding the presence of this enzyme.

Purified glutathione synthetase catalyses an exchange of $[\text{P}^{32}]\text{ADP}$ with ATP in the absence of any component but Mg^{2+} (Snoke & Bloch, 1955), and might thus have contributed to the exchange at present observed. However, the purified glutathione synthetase catalysed also a synthesis of ATP from ADP, $[\text{P}^{32}]\text{P}_i$ and glutathione (Snoke & Bloch, 1955). With the present microsomal fraction, the addition of glutathione (32 mm) did not produce $[\text{P}^{32}]\text{ATP}$ from $[\text{P}^{32}]\text{P}_i$ in a reaction mixture (see the Experimental section) that contained ADP and ATP. Further, glutamine synthetase, which occurs in cerebral microsomal fractions (Salganicoff & de Robertis, 1965), was also excluded since no significant formation of labelled ATP occurred from $[\text{P}^{32}]\text{P}_i$ in the presence of glutamine (15 mm), which is a sensitive test for the presence of the enzyme (Levintow & Meister, 1954).

DISCUSSION

The microsomal preparations have thus been shown to contain three categories of enzymes

Table 4. $[\text{C}^{14}]\text{ADP-ATP-exchange}$, adenylate-kinase and sodium-plus-potassium ion-stimulated adenosine-triphosphatase activities of cerebral microsomal fractions

The original and the MgATP -washed microsomal preparations were as described in Table 2, and the ribonuclease treatment was as described in Fig. 2.

Material	$(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity ($\mu\text{moles of P}_i$ formed/mg. of protein/hr.)	$[\text{C}^{14}]\text{ADP-ATP-}$ exchange activity ($\mu\text{moles of } [\text{C}^{14}]\text{ADP}$ exchanged/mg. of protein/hr.)	Adenylate-kinase activity ($\mu\text{moles of } [\text{C}^{14}]\text{ADP}$ formed/mg. of protein/hr.)
Original microsomes	27.9 (3)	19.1 (3)	8.0 (3)
Washed microsomes	21.9 ± 4.5 (5)	3.06 ± 0.83 (5)	0.88 ± 0.59 (5)
Washed microsomes after ribonuclease treatment (20 min.)	15.7 (2)	0.81 (2)	0.24 (2)

reacting with ATP. The two most active are: (i) the readily extracted ADP-ATP-exchange activity and (ii) the difficultly extracted (Na⁺,K⁺)-activated ATPase. In addition (iii) there remain, associated with the ATPase, exchange systems of less activity that are difficult to extract and due to more than one enzyme. Comment on systems (i)–(iii) follows.

(i) *Extracted [¹⁴C]ADP-ATP-exchange system.* The enzyme or enzymes now newly extracted and partially purified catalyse reaction (1):

Expressing the conversion with an exchange enzyme E¹ as a reactant (more than one such enzyme may be involved) gives reactions (2) and (3) as its most likely course:

for the reaction proceeds rapidly without loss of ATP, and the preparation does not catalyse exchange of [³²P]P_i with ATP. The conclusion of Skou (1960), Albers *et al.* (1963) and Post *et al.* (1965) that microsomal enzymes catalysing reactions (1)–(3) were also ATPases by virtue of reaction (4):

(or equivalent reactions, with or without specifying the participation of cations or water) is disproven with the present preparation. This has been obtained without ATPase activity, and without major loss of the ATPase with which it was formerly associated. Thus the extracted exchange reaction does not appear to be a modified or degraded moiety of the enzyme measured by the ATPase assay; other possible relationships between the two activities are discussed below.

(ii) *Sodium - plus - potassium ion - stimulated adenosine triphosphatase.* The present investigations also alter accepted opinions about the (Na⁺,K⁺)-ATPase (E²) of cerebral microsomes, the action of which may be expressed as reaction (5):

On the basis of actions of activators and inhibitors, Skou (1964) regarded 'the same enzyme system as responsible for the two reactions', i.e. concluded E¹ and E² to be identical or overlapping. On this basis he postulated intermediate steps in the ATPase equivalent to reactions (6) and (7):

Comparable relationships between ATPase and exchange reactions, and a similar intermediate, were postulated with respect to a kidney preparation that also catalysed both reactions, by Post & Sen (1965), who included it as one of several components in an active-transport mechanism. Again, in a particulate fraction from the electric organ of *Electrophorus electricus*, Albers *et al.* (1963) found the two reactions and again regarded them as a property of a single enzyme system. Albers *et al.* (1963) gave as an additional support for this relationship the similarity in the rates with which a given preparation catalysed the two reactions. We consider, however, that such similarity allows only limited conclusions; indeed, our starting material also showed this property (Table 1), but in the final washed preparation, which retained almost all the ATPase, reaction (5) proceeded at 30 times the speed of reaction (1).

If reaction (5) indeed included steps (6) and (8):

with E² but otherwise analogous to reactions (2) and (3) then it would be necessary for reaction (6) to proceed from left to right at a speed equal to or greater than the speed of reaction (5). In the final washed preparation, such a velocity for reaction (6) would be compatible with the observed speed for the exchange only if reaction (8) proceeded from left to right at no more than 3% of the speed of reaction (6). Conceivably, withdrawal of E² ~ P_i by reaction (7) could have this effect.

It may be noted that myosin ATPase is found not to catalyse exchange of either ADP or P_i with ATP (Koshland, Budenstein & Kowalsky, 1954) but nevertheless affords evidence for both a myosin-ATP complex and a phosphorylated myosin as intermediates (Yount & Koshland, 1963; Tokiwa & Tonomura, 1965). With the present microsomal system it is also to be concluded that, if a compound E² ~ P_i is intermediate in the (Na⁺,K⁺)-ATPase, this rapid withdrawal by reaction (7) is a property which distinguishes it sharply from E¹ ~ P_i.

(iii) *Residual ADP-ATP - exchange activity.* Major contribution to the non-extracted exchange activity of the microsomal residue is made by an adenylate kinase, but very little by the nine other systems specified in the Results section. The enzymic identity of the unextracted ADP-ATP-exchange activity is uncertain. There is no longer, however, a basis for considering that the ATPase normally catalyses appreciable exchange and in this respect it resembles myosin and actomyosin.

It has been noted above that the residual exchange activity is affected little if at all by concentrations of Na⁺, K⁺ and ouabain to which the (Na⁺,K⁺)-ATPase is susceptible. The present

washed microsomal fraction thus differs in this respect from the nerve preparation obtained by Skou (1960), who reported inhibition by Na^+ ; and also that, in the presence of Na^+ , K^+ also inhibited whereas ouabain partly antagonized these inhibitions. Each of these actions reported for Na^+ , K^+ and ouabain on the nerve preparation is the converse of the actions of the reagents on the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ that was also present. The effects are thus more simply explained in terms of removal of ATP, or dilution of labelled ADP as a result of ATPase action. That such effects were not found in the present experiments may depend on differences in the tissue investigated, or in the substrate, substrate concentrations and temperature employed.

(iv) *Interrelations*. It is of possible importance that our initial microsomal preparation shows also the similarity in rate of ATPase and exchange reactions reported by Albers *et al.* (1963) in quite different preparations. As we have shown that at least 96% of the cerebral microsomal ADP-ATP exchange is not due to the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$, we reinterpret this regularity to suggest that the exchange activity may function in some association with the ATPase. The enzymes at present known by exchange abilities may be judged to cause net change in substrates at present unspecified. The ATPase may itself be complex, especially in its microsomal milieu. Thus reaction between the microsomal fraction and MgKATP appeared to yield a labile intermediate, possibly an acyl phosphate, before it yielded P_i (Hems & Rodnight, 1965; Nagano *et al.* 1965; for analogous findings in other systems see Whittam, Wheeler & Blake, 1964; Post *et al.* 1965). The present observations, which widen the scope of microsomal interaction with ATP, emphasize the need for caution in ascribing multiple effects to a single enzyme system.

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