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Microsomal ATPase of rabbit brain and effects of general anesthetics

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IN RECENT years an interaction of Na^+- and K^+-activated, Mg^{2+}-dependent adenosine triphosphatase (Na^+K^+-ATPase) and cation translocation at excitable cell membranes has been well established.[1] The present study was indertaken to investigate a possible effect of general anesthetics on ouabain-sensitive Na^+K^+-ATPase of microsomal fractoins of rabbit brain.

Cerebral microsomal fraction of rabbit were prepared according to the method of Skou.[2] Rabbits were sacrificed by injection of air into an ear vein and the brain rapidly removed and chilled in iced 0·25 M sucrose with 0·03 M histidine buffer. After removal of the brain stem, the cerebrum was homogenized in a Potter–Elvehjem glass homogenizer fitted with a Teflon pestle, with 7 vol. of 0·25 M sucrose containing 0·03 M histidine, 0·005 M EDTA, and 0·1% deoxycholic acid, adjusted to pH 6·8 with Tris buffer. After centrifugation at 8000 *g* for 15 min, the supernatant fraction was again centrifuged in a Hitachi 40-P ultracentrifuge* at 59,300 *g* for 1 hr at 0°. The packed sediment was resuspended in half the original volume of histidine buffer containing 0·25 M sucrose, 0·03 M histidine base, and 0·001 M EDTA free of sodium, and the pH adjusted to 6·8 with Tris. The microsomal fraction was divided into small quantities and kept frozen until use. Significant loss of activity was not observed after several weeks, when the preparation was stored at −20°. Thawed material was used in the experiments.

The ATPase activity was measured in 1·2 ml of buffer solution with appropriate concentration of Tris-ATP and $MgCl_2$ and with or without 100 mM NaCl and 20 mM KCl; pH was again adjusted to 7·6 with Tris buffer. Sodium-free Tris-ATP was prepared by passing disodium ATP (Calbiochem) through a column of Amberlite IR 120 cation-exchange resin. After warming the ATP-containing solution in a glass-stoppered test tube for 5 min at 38° in a Dubnoff shaker, 100 μl of the microsomal fraction was introduced. The reaction was stopped at an appropriate time by addition of trichloroacetic acid.

Liberated inorganic phosphate was determined by a modification of the method of Fiske and SubbaRow,[3] which consisted of extraction of inorganic phosphate into isobutanol; ascorbic acid was used as the reducing agent. Activity was expressed as inorganic phosphate liberated per unit time, per 1 mg enzymatic protein. Enzymatic protein was determined by the biurett method described by Layne.[4]

Hydrolysis of ADP was determined in a similar fashion, replacing ATP with ADP. ADP (Sigma) was used as substrate without further purification. The activity was measured in the presence of Na^+ and K^+. A possible contamination of the preparation with adenylate kinase was determined

* Hitachi Co. Ltd., Tokyo, Japan.

by heating the microsome to 70° for 1 min. The heating procedure destroyed ATPase activity. Liberation of ATP from ADP in the heat-denatured microsome was studied under the same conditions of release of inorganic phosphate from ADP. After incubation for 30 min at 38°, the reaction mixture was cooled, and the ATP content was measured by means of firefly bioluminescence.[5]

Diethylether and halothane were vaporized in a copper kettle with oxygen, and concentrations were estimated according to temperature and the diluent flow of oxygen. Anesthetics were bubbled through ATP-containing solution in a glass-stoppered test tube before the addition of cerebral microsome. Concentrations of anesthetics were expressed as partial pressure in the gas phase equilibrated with the test solution.

The microsomal fraction contained ouabain-sensitive Na^+K^+-ATPase as shown in Fig. 1. About 6-fold activation was observed by the addition of Na^+ and K^+ when compared to the activity with

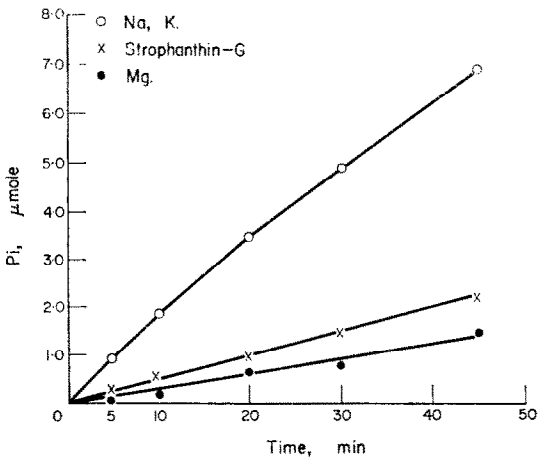

FIG. 1. Time-dependent ATPase activities as expressed by inorganic phosphate (Pi) liberated /1 mg enzymatic protein. The reaction mixture shown in the upper line contained 3 mM ATP, 6 mM Mg^{2+}, 100 mM Na^+ ,and 20 mM K^+. The middle line shows suppression by the addition of 10 μM strophanthin-G to the above mixture. The reaction mixture expressed by the lower line contained 3 mM ATP and 6 mM Mg^{2+} without Na^+ and K^+.

Mg^{2+} alone. Release of inorganic phosphate from ADP is shown in Fig. 2. We found that about 1·8 μmole ADP were hydrolyzed per 30 min/1 mg enzymatic protein. When heat-denatured microsome was incubated with 3 mM ADP for 30 min at 38°, 0·5 μmole ATP/1 mg enzymatic protein was detected when determined by the initial flash height of luciferin bioluminescence. Although the

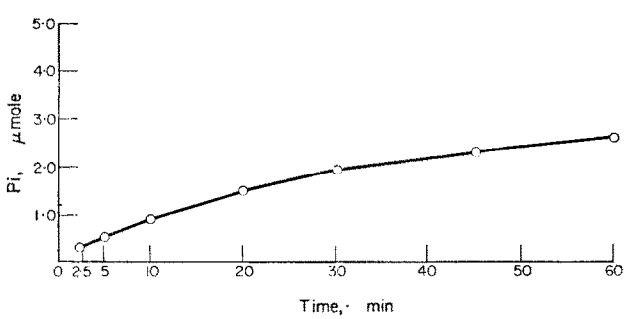

FIG. 2. Time-dependent inorganic phosphate release from 3 mM ADP with 100 mM Na^+, 20 mM K^+, and 6 mM Mg^{2+}. Activity is expressed as μmole Pi released/1 mg enzymatic protein.

possibility that ADP was hydrolyzed by the microsome directly could not be eliminated, the discrepancies between the values obtained by measurement of inorganic phosphate release and luciferin bioluminescence might be caused partly by the inactivation of adenylate kinase by heat denaturation and partly by feedback inhibition imposed on the enzyme by the accumulation of ATP.

The effects of diethylether and halothane on the activity of ATPase are shown in Figs. 3 and 4. The incubation mixture contained 3 mM ATP, 100 mM Na^+, and 20 mM K^+ together with 6 mM

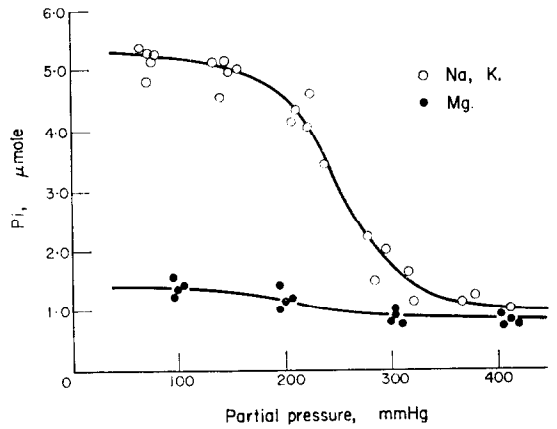

FIG. 3. Effect of diethylether. Open circles: medium contained 3 mM ATP, 6 mM Mg^{2+}, 100 mM Na^+, and 20 mM K^+. Black circles: medium contained 3 mM ATP and 3 mM K^+ without Na^+ and K^+. Activity is expressed as μmole Pi released/1 mg enzymatic protein per 30 min. Concentrations of diethylether are expressed by partial pressure in the gas phase, equilibrated with the reaction mixture.

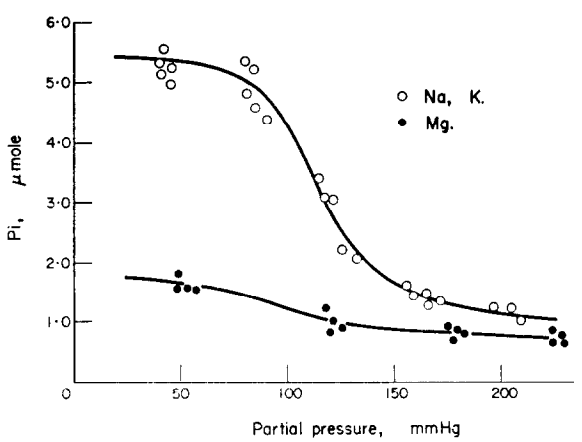

FIG. 4. Effect of halothane. Open circles: medium ocontained 3 mM ATP, 6 mM Mg^{2+}, 100 mM Na^+, and 20 mM K^+. Black circles: medium contained 3 mM ATP and 3 mM Mg^{2+} without Na^+ and K^+. Activity is expressed as μmole Pi released/1 mg enzymatic protein per 30 min. Concentrations of halothane are expressed by partial pressure in the gas phase, equilibrated with the reaction mixture.

Mg^{2+}. A decline in activity of ATPase was demonstrated when the partial pressure of diethylether exceeded 200 mm Hg; there was no demonstrable suppression of ATPase activity when the partial pressure of diethylether was in the clinical range. With halothane, inhibition also became evident

when the partial pressure exceeded 100 mm Hg, also in excess of the clinical range. The tension of anesthetics showing apparent inhibition was roughly ten times that of clinical concentrations. When the reciprocal of the reaction velocity was plotted against the reciprocal of substrate concentration, a non-competitive type of inhibition was demonstrated with both anesthetics, as shown in Figs. 5 and 6. Effects of halothane and diethylether on Mg^{2+} activated-ATPase which does not

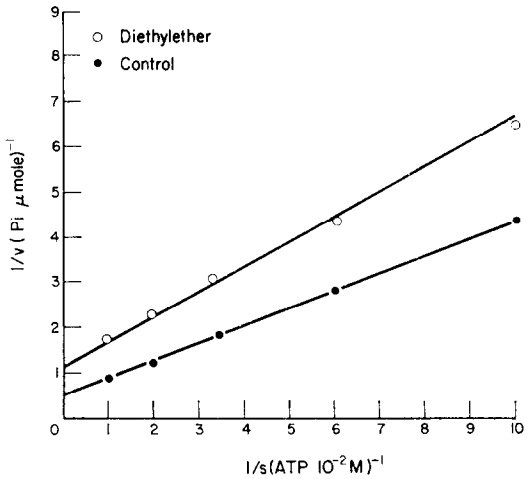

FIG. 5. Lineweaver–Burk plot of inhibition of ATPase by diethylether. The partial pressure of diethylether was 260 mm Hg. Reaction mixture contained 100 mM Na^+, 20 mM K^+, and 6 mM Mg^{2+}, with varying concentrations of ATP. The concentrations of ATP used were 10 to 1 × 10^{-3} M. Reaction velocity is expressed as μmole Pi released/1 mg enzymatic protein per 30 min.

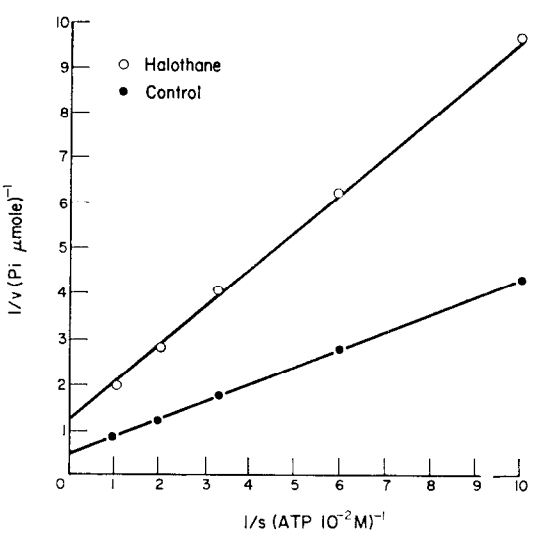

FIG. 6. Lineweaver–Burk plot of inhibition of ATPase by halothane. The partial pressure of halothane was 130 mm Hg. The experimental conditions were as shown in Fig. 5.

require Na^+ and K^+ were also examined and are shown in Figs. 3 and 4. Only a slight decrease of activity was observed.

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Department of Anesthesiology,
The Hospital of National Cancer Center,
Tsukiji 5-Chome, Chuoku, Tokyo, Japan

ISSAKU UEDA
WATARU MIETANI

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Stereoselective metabolism of amphetamine*

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SINCE *d*-amphetamine is a potent pharmacological agent—central stimulant, pressor agent and appetite depressant—whereas *l*-amphetamine is inert (or very low in activity), a difference in the fate of the stereoisomers may help to define the biochemical processes that determine the drug's actions. Gunne[1] has presented indirect evidence of stereoselectivity in amphetamine metabolism in man.

Direct evidence for a specific stereoselective pathway, utilizing the amphetamine metabolite *p*-hydroxyamphetamine (α-methyl tyramine), has been demonstrated *in vivo* in rats by Goldstein and Anagnoste.[2] The initial steps in this pathway are hydroxylations at two different positions. The first, hydroxylation of amphetamine at the *para* position on the benzene ring to yield *p*-hydroxyamphetamine, does not discriminate between stereoisomers and is thought to occur in the liver. The second, and stereoselective, step is hydroxylation of *p*-hydroxyamphetamine at the β-carbon of the side chain to yield *p*-hydroxynorephedrine (α-methyl octopamine). This reaction proceeds only for the *d*- and not for the *l*-isomer of hydroxyamphetamine. It is probably catalyzed by dopamine-β-oxidase, an enzyme located in the catecholamine-containing granules of sympathetic nerves.[3, 4] Although the stereoselectivity of the β-hydroxylation has so far been demonstrated in rats only, the β-hydroxylating step has been shown to occur *in vivo* in man,[5] in rats,[6, 7] and in cats;[8, 9] *p*-hydroxylation has been described in rats, dogs and man,[10–12] and in cats.[9]

The present work was designed to study the differential metabolism of amphetamine isomers as revealed by the presence of optically active metabolites in urine. Rats that had been given racemic *d*, *l*-amphetamine were found to excrete less *d*-than *l*-hydroxyamphetamine during the time intervals examined.

Racemic *p*-hydroxyamphetamine hydrobromide and dextro-*p*-hydroxyamphetamine hydrobromide were provided by Smith, Kline & French Laboratories. Trifluoroacetyl-*l*-prolyl chloride was synthetized by Dr. Enoch Gordis according to the method of Weygand *et al.*[13] Amphetamine sulfate injection, USP, and dextro-amphetamine sulfate injection were obtained from Gotham Pharmaceutical Co.; *l*-amphetamine sulfate was obtained from Walker Chemicals. The β-glucuronidase was obtained from Warner-Chilcott.

Twenty male albino rats weighing about 250 g were given 5 mg *d*, *l*-amphetamine i.p. per kg. Eight rats were given 2·5 mg of either *d*- or *l*-amphetamine i.p. per kg. Urine was collected for varying intervals and extracted for hydroxyamphetamine by a modification of the method of Axelrod.[10] After incubation with a commercially available glucuronidase at 37° for 48 hr, the urine was washed with ethyl ether at pH 4·5 until the ether phase was clear, then saturated with sodium chloride and

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