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Acetylcholine depletion of synaptic vesicles from guinea pig brain

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NEUROCHEMICAL transmitters such as acetylcholine(ACh) and monoamines(noradrenaline, dopamine and 5-hydroxytryptamine) are known to be present in the synaptic vesicle fraction.[1,2] However, there is still little known about the ACh in synaptic vesicles. One interesting problem is the form in which it is present in isolated vesicles.

In the present work a preparation of synaptic vesicles was obtained using DEAE-Sephadex column chromatography[3,4] and its ACh content was measured at various temperatures, pH values and ionic strengths. Similar experiments were performed with another preparation of synaptic vesicles obtained from a crude preparation(P_5-fraction) without DEAE-Sephadex column chromatography. ACh was measured by bioassay.

Methods

A preparation of synaptic vesicles consisting of both coated and smooth synaptic vesicles was obtained as reported previously.[3,4] This crude preparation(P_5-fraction) was applied to a column of DEAE-Sephadex. The column was eluted with 500 mM KCl and a fraction of the eluate was collected (E-fraction). Another preparation was obtained as follows. A suspension of the P_5-fraction was centrifuged at 30,000 *g* for 10–20 min to obtain the supernatant fraction(SS-fraction). This centrifugation should precipitate the heavier, coated vesicles from the mixture of smooth synaptic vesicles and coated vesicles.

These fractions were incubated in 40-P tubes of a Beckman ultracentrifuge unless otherwise stated. The incubation mixture contained 5 ml of SS- or E-fraction, 20 mM tris–maleate buffer, pH 6·5 and other additions as indicated. The final volume was adjusted to 5·5 ml with the SS-fraction and to 7·25 ml with the E-fraction using deionized water. After incubation under various conditions, the mixtures were centrifuged at 100,000 *g* for 1 hr. The resulting precipitates were resuspended with 1–2 ml of 10 mM acetate buffer, pH 4·0, and ACh was extracted by boiling the mixtures at 100° for 10 min.[5] The mixtures were then centrifuged in a clinical centrifuge, and the resulting supernatant was used for bioassay of ACh with isolated guinea pig ileum.[6] Usually the threshold response for ACh was obtained with 6×10^{-10}–1×10^{-9} g/ml of ACh chloride in the Magnus bath apparatus. Values for ACh are expressed as ACh chloride. To identify ACh, blockade or loss of muscle response to ACh was tested by addition of atropine sulfate(5×10^{-8} g/ml) or erythrocyte acetylcholinesterase (EC 3.1.1.7) to a neutral extract of ACh.

Results

Effect of time. Samples of mixtures containing the SS-fraction were centrifuged at intervals during incubation at 0 or 20° and the amount of ACh remaining in the precipitate was estimated [Fig. 1(a)]. At 20° the ACh content of the precipitate decreased to about 75 per cent of the initial value in about 10 min, and reached a steady level in 15 min. At 0°, the ACh content was not decreased appreciably even after incubation for 1 hr.

Effect of temperature. Figure 1(b) shows the effect of temperature on the ACh retained in the precipitate of the SS-fraction on incubation for 15 min at pH 6·5. The ACh decreased to about 60 per cent of the initial value at 20°, and was lost completely at 50°. Thus increase in temperature resulted in more rapid loss of ACh from the synaptic vesicles of the SS-fraction.

Effect of pH. As shown in Fig. 2, ACh retention by the SS- and E-fractions was similar in media at different pH values. There was a broad peak of retention at pH 5·5–6·5 (Fig. 2). In more acidic media the ACh content decreased rapidly, while in more alkaline media it decreased more gradually.

Effects of various ions and ionic strengths. As shown in Fig. 3, the ACh content of the precipitate of the SS-fraction decreased gradually with increase in the concentration of added salts. No specific effects were observed with NaCl, KCl, $CaCl_2$ or $MgCl_2$ at various concentrations below the iso-osmotic concentration. Increase in the ionic strength from zero to 1·0 resulted in decrease in the ACh content to 60 per cent of the initial value.

When 1 mM EDTA or glycoether-ethylenediamine-tetraacetic acid (GEDTA) was added to the P_5-fraction the ACh content of the SS- and E-fractions decreased. The decrease in the ACh content

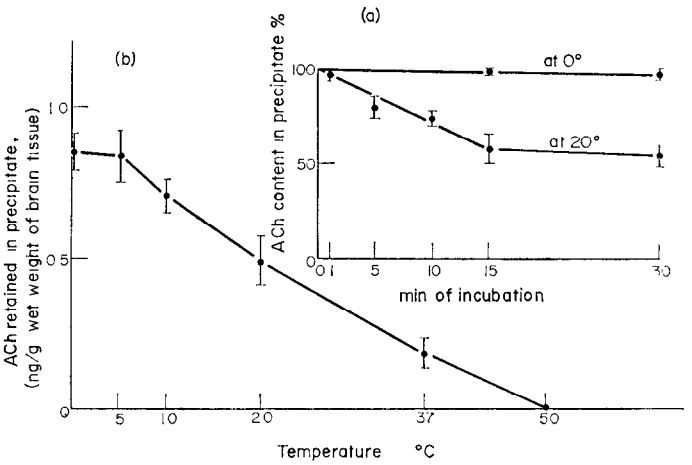

FIG. 1. Effects of time and temperature on ACh retention in the precipitate from the SS-fraction. (a) Retention of ACh at pH 6·5. The ACh content is expressed as a percentage of that at zero time. The initial content was about 1·1 ± 0·1 ng/g wet weight of brain tissue. Each point represents the mean of three determinations. Vertical lines represent S.E.M. (b) Retention at different temperatures at pH 6·5. Incubations were carried out for 15 min in the medium described in the text. Each point represents the mean of three determinations. Vertical lines represent S.E.M.

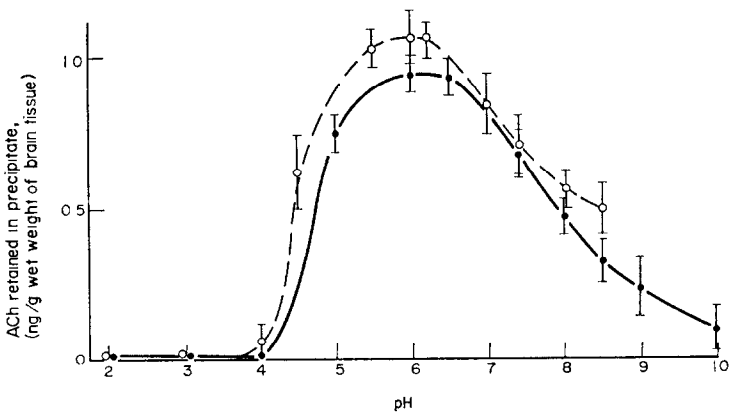

FIG. 2. Effect of pH on ACh contents of precipitates of the E- and SS-fractions. Media at various pH values were obtained by addition of 20 mM tris–maleate buffers of various pH's. SS-fraction incubated at 20° for 10 min (—●—). E-fraction incubated at 0° for 60 min (- -○- -). Each value is the mean of those of at least four experiments. Vertical lines represent S.E.M.

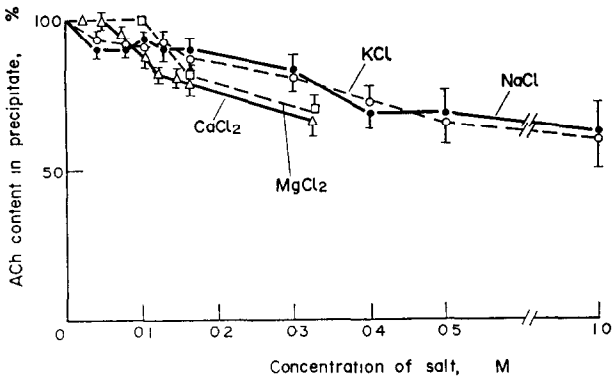

FIG. 3. Effects of various concentrations of salts on the ACh content of the precipitate of the SS-fraction. The incubation mixtures contained SS-fraction, 20 mM tris–maleate buffer, pH 6·5, and salts at ionic strengths of 0–1·0. NaCl (—●—), KCl (– –○– –), $CaCl_2$ (—△—), $MgCl_2$ (– –□– –). Incubations were carried out at 20° for 10 min. The ACh content of the SS-fraction without added ions was defined at 100 per cent. Other values are expressed as percentages of the control values. The average control value was 1·0 ± 0·1 ng/g wet weight of brain tissue. Each point represents the mean of three determinations. Vertical lines represent S.E.M. Absence of vertical line indicates that the standard error is smaller than the symbol used. (Half-lines are used for some values to avoid overlap.)

caused by these chelators was not antagonized by the addition of 20 mM $MgCl_2$ or $CaCl_2$. Thus these chelators probably release ACh from synaptic vesicles by some mechanism not involving chelation.

Discussion

Figure 1(a) and (b) suggest that the amount of ACh retained in the synaptic vesicles depends on the temperature. These data are essentially consistent with those of Barker and co-workers.[7] Synaptic vesicle fractions isolated by different methods all showed similar tendencies.

In the present experiments on the effect of ionic strength, none of the ions tested seemed to have a specific effect on the membrane of the synaptic vesicles. Takeno and co-workers demonstrated that the quantity of ACh released from the vesicle fraction isolated by their method was not affected by the kind of ion used on incubation at 0° for various times.[8] Kuriyama and co-workers, on the other hand, reported that [^{14}C]ACh binds ion-sensitively to a purified vesicle fraction from whole mouse brain.[9] However, the interaction of ions with the membrane of synaptic vesicles requires further study.

From the pH–ACh–retention curves obtained (Fig. 2) some ionizing groups in the membrane of the synaptic vesicles seem to regulate the retention of ACh. Rapid loss of ACh from the synaptic vesicles at low and high pH values is probably simply due to destruction of the vesicles, involving structural changes of the inner and outer membranes of synaptic vesicles which retain ACh.

As previously reported, on centrifugation of the E-fraction, coated vesicles were precipitated to the bottom of the centrifuge tube, smooth synaptic vesicles being precipitated above them.[3,4] The smooth synaptic vesicles are probably the predominant vesicular structures in the SS-fraction, since this is the supernatant fraction obtained by centrifuging the E-fraction at 30,000 *g* for 20 min.

From the present results and discussion it seems likely that the smooth synaptic vesicles are more important for carrying ACh than the coated vesicles in the nerve ending fraction of guinea pig brain.

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The level of pyridoxal phosphate (PLP) in the blood plasma of rats exposed to carbon disulphide

The mechanism by which carbon disulphide brings about metabolic disturbances is not clearly understood. Some authors[1] suggested that interaction between carbon disulphide and vitamin B_6 might represent the basic primary site of action, and some indirect observations seemed to support this hypothesis.[1-3]

In this experiment an attempt was made to obtain direct proof for the interaction *in vivo* between carbon disulphide and the most essential component of vitamin B_6, pyridoxal phosphate.

Experimental

The experiments were performed on white female rats of the Wistar strain, body weight about 200 g, fed standard LSM diet.* The animals were exposed to carbon disulphide in two groups differing in the kind of exposure:

(a) *Long-term exposure.* A dynamic experimental chamber was used into which the CS_2 vapours were introduced automatically at a constant rate.[4] The exposure lasted for 6 months, 6 days a week, 5 hr a day. The concentration of CS_2 in air was measured colorimetrically[5] and ranged from 1·5 to 1·8 mg/l. The rats were fed *ad lib.* throughout the experiment. The exposure started when rats were 3 months old.

(b) "*Continuous*" *exposure.* Rats were kept in a small dynamic chamber[6] continuously for 2 days, the concentration in air being 0·8–1·2 mg/l. All rats (experimental and control) were fasted during time of exposure. The animals used were 5 months of age.

For both above groups controls were also studied. These were subjected to the same conditions, but were not exposed to carbon disulphide.

In both groups animals were killed by decapitation immediately after exposure. The blood plasma was obtained as described by Wachstein *et al.*[7] The determination of pyridoxal phosphate (PLP) was based on its coenzyme function in a tyrosine decarboxylase system, as described by Boxer *et al.*,[8] and modified later by Wachstein *et al.*,[7] using the apoenzyme of tyrosine decarboxylase (Sigma Chem. Co.). An internal standard was used for the calculation of results.

Results and discussion

The results presented in Table 1 show a distinct decrease of PLP in the blood plasma of rats exposed to CS_2. The drop in the level of PLP in exposed animals was roughly 60 and 75 per cent of the control values, for chronic long-term exposure, and 2 days continuous exposure of rats, respectively.

* Standard diet for rats and mice (containing 0·03 mg vitamin B_6/100 g diet) manufacturer: Wytwórnia Pasz, Łowicz.