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RELEASE OF ACETYLCHOLINE FROM ISOLATED SYNAPTIC VESICLES—I.

METHODS FOR DETERMINING THE AMOUNT RELEASED*

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Abstract—Methods have been developed which permit the determination of the rate of spontaneous release of acetylcholine from isolated synaptic vesicles. After isolation and harvesting, the synaptic vesicle fraction is divided into several 1-ml samples and incubated for various times and temperatures. The incubation is terminated by addition of 50 units of acetylcholinesterase, followed within 5 sec by 0·2 N HCl in an amount sufficient to decrease the pH to 4. The added enzyme serves the purpose of destroying 'free' acetylcholine, and decreasing the pH to 4 inactivates the added enzyme. The acetylcholine surviving this treatment is considered 'bound'. Zero time values were obtained as described above by treating a sample immediately after isolation. This value was defined at 100 per cent and all other values are expressed as per cent of the zero time. Experiments were performed at 0°, 20°, and 37°. After 20 min of incubation, no acetylcholine (ACh) was released at 0°; at 20°, 55 per cent was released; and 37°, 90 per cent was released. The release of ACh from isolated synaptic vesicles is temperature dependent, and the absolute rate of release in nanograms/sec is proportional to the initial amount bound. The method described in this paper has the advantage of permitting determination of both the initial (rapid) and later (slow) phases of release.

IN RECENT years several drugs have been shown to act presynaptically either on the synthesis, storage or release of transmitter agents.[1, 2] Technical advances in the isolation of neural subcellular particles[3] have permitted pharmacological investigations to be conducted on isolated nerve endings and transmitter storage particles. Most of these investigations have been conducted *in vitro* on the adrenergic nerve endings and storage particles and have been the subject of recent reviews.[4, 5] To date there have been relatively few pharmacological investigations on isolated cholinergic synaptosomes (pinched-off nerve endings). Whittaker[6] and Takeno *et al.*[7] have reported on the effects of various ions, enzymes, and physical treatments on the storage and release of acetylcholine (ACh) from synaptosomes. De Robertis *et al.*[8] have shown selective binding of ^{14}C-D-tubocurarine to cholinergic nerve-ending membranes. It has been demonstrated that chlorpromazine (CPZ) prevented release as well as uptake of ACh by synaptosomes.[9]

The paucity of pharmacological studies on isolated cholinergic synaptic vesicles appears to be due mainly to the lack of suitable methods for studying the release, storage or uptake phenomena. Whittaker *et al.*[10] reported methods for the isolation

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of synaptic vesicles and commented briefly on factors affecting the spontaneous release of ACh from the vesicles, but did not elaborate on the methods used for studying the release. Therefore, before any pharmacological investigation could be undertaken, it was necessary to develop methods for studying the release of ACh from isolated synaptic vesicles.

In this paper we shall describe a method which permits the determination of ACh remaining bound to isolated synaptic vesicles after various treatments and present data on the effect of temperature on the spontaneous release of ACh.

METHODS

Materials. Acetylcholine chloride and eserine salicylate were purchased from Merck & Co. and bovine erythrocytic acetocholinesterase (AChE) from Sigma.

Isolation of synaptic vesicles. All manipulations involving the isolation and initial treatment of the synaptic vesicles were performed at 0–4°. Synaptic vesicles were isolated from cerebral cortical tissue of 5 guinea pigs (approx. 8·7 g) by the abbreviated method of Whittaker *et al.*,[10] with the following minor modifications. Sucrose solutions were prepared in double-distilled water and buffered with maleate–NaOH buffer at pH 6·9 (final concentration of maleate was $1–2 \times 10^{-4}$ M). The 0·32 M sucrose solutions and the double-distilled water used for the osmotic shock of the crude mitochondrial pellet were 10^{-5} M with respect to eserine salicylate. The second pellet was washed twice in 0·32 M sucrose. The first repelleting of the synaptosomal fraction was at 12,000 *g* for 20 min and the second at 15,000 *g* for 30 min. This was followed by osmotic shock.

Bioassay. Four- to six-point assays, depending on the concentration of ACh-like activity, were performed on the isolated guinea pig ileum. The injection volume of the unknowns was limited to 0·15 ml; therefore, when the yield of ACh was low, 4-point assays were used. Contractions were recorded by means of a Grass force-displacement transducer (FT03) and a Grass model 7 polygraph. Morphine sulfate, 40 mg/l., was included in the Tyrode solution used for bathing the ileum to inhibit spontaneous activity.

The following criteria were used to establish that the biological activity measured was ACh or a closely related choline ester. The activity was abolished by treating the ileum with atropine sulfate (1 mg/l. of Tyrode solution) or by the addition of AChE to a neutral (pH 7) vesicle suspension which had been treated to release all bound ACh. The biological activity was lost upon heating the vesicle suspension for 5 min at 100° after the pH had been adjusted to 9. Conversely the activity was stable when the suspension was heated at pH 4. Addition of eserine (4 μg/ml) to the vesicle suspension or the Tyrode solution potentiated the activity. Also, an index of discrimination[11] of 1 was obtained from parallel ACh assays by using the isolated guinea pig ileum and the eviscerated cat blood pressure preparation of MacIntosh and Perry.[12]

Determination of release. Since the release of ACh from its storage particle involves a transition from bound to free ACh, it should be possible to develop a method for studying the release by following an increase in free ACh or a decrease in bound ACh. The converse would be true for studying the uptake of ACh by synaptic vesicles. Determination of the amount of ACh remaining bound to the synaptic vesicles after various periods of incubation will allow calculation of the amount of ACh released

from synaptic vesicles. This can be achieved by the selective destruction of free ACh by AChE, since bound ACh may be defined as that ACh resistant to hydrolysis by AChE. The amount of free ACh present at any time during the incubation period is very small in relation to the hydrolytic activity of the added AChE, and is therefore assumed to be rapidly hydrolysed upon the addition of the enzyme. The added enzyme is inactivated immediately by decreasing the pH of the suspension to about 4·0. It is very important that the enzyme be rapidly inactivated, because release occurs continually, but at a slower rate than the enzymic hydrolysis of ACh. Hence, as soon as any ACh is released, it is hydrolysed.

Immediately after the isolation of the synaptic vesicles, the fraction was divided into several 1-ml samples, usually 12–15 ,and incubated at 0°, 20°, or 37° for various lengths of time. The incubation period was terminated by the addition of 50 units of AChE contained in 0·03 ml of 0·9% saline and 0·1% gelatin solution. The seemingly large amounts of AChE were employed because eserine was present in the sucrose solutions used in the isolation of the synaptic vesicles. Control experiments established that the added enzyme was effective in spite of the presence of eserine. The addition of AChE was followed within 5 sec by sufficient amounts of 0·2 N HCl to decrease the pH to 4·0. Usually the volume of HCl required was between 0·01 and 0·02 ml and had to be determined for each experiment. One or two of the samples were used for this purpose. Also as Whittaker *et al.*[10] have reported, decreasing the pH of the synaptic vesicle suspension to 4·0 caused complete release of bound ACh in about 2 min.

Control experiments were designed to assess the following: (1) effect of the AChE preparation on the release of ACh; (2) time course of ACh hydrolysis by AChE added to the vesicle fraction; and (3) time course for inactivation of the added AChE These are described below.

Effect of AChE *on* ACh *release.* AChE (750 units) in 0·5 ml of saline–gelatin vehicle was inactivated by adjusting the pH to 4·0 and then neutralized with 0·2 M Tris (volume changes did not exceed 1 per cent). A volume of 0·21 ml of the neutralized, inactivated enzyme solution was added to 7·0 ml of the synaptic vesicle suspension and carried through the experimental procedure described above. A similar experiment was performed with the saline–gelatin vehicle. Experiments of this nature were performed at 20° and 37°.

Time course of ACh *hydrolysis.* Several 1-ml samples of a freshly isolated synaptic vesicle fraction were heated at 50° for 5 min after the pH had been decreased to 4·0 to insure complete release of bound ACh. Bioassays of samples so treated established that 100 per cent of the bound ACh had been released. After releasing all bound ACh, the pH was adjusted to 6·8. AChE (50 units) was then added to each sample and this was followed in 3 or 5 sec by the addition of sufficient 0·2 N HCl to decrease the pH to 4. The resulting preparations were then bioassayed for ACh. Control experiments of this type were performed at 0°, 20°, and 37°.

Time course of enzyme inactivation. From the above, the volume of 0·2 N HCl required to decrease the pH of 1 ml of the vesicle fraction to 4 was determined. This amount of HCl was added to several 1-ml samples of the same vesicle fraction as that used above. AChE (50 units was then added to samples at 0°, 20°, or 37°. The resulting pH was 4·0. Samples treated in this fashion were bioassayed and compared to samples which did not receive AChE.

The samples treated as described above were bioassayed the same day or stored at −20° and assayed the following day.

Electron microscopy of various cell fractions was done according to the method described by Verster *et al.*[13]

RESULTS AND DISCUSSION

Other approaches were tried in preliminary experiments attempting to determine the release of ACh from isolated synaptic vesicles. There were: incubation of the vesicle suspension for various times at various temperatures, pelleting the vesicles at 100,000 *g* for 60 min after the suspension had been diluted 1:1 with distilled water, and assaying the supernatant for ACh; bioassaying the vesicle suspension at various times after isolation and during the incubation in hope that only the free ACh would cause contractions; and ultrafiltration of the vesicle suspension by means of a Diaflo model 50 cell with a UM-I membrane and assaying the filtrate collected at various time intervals for ACh. For reasons presented below, none of these methods was found to be useful.

Regardless of the time period of incubation, 90–100 per cent of the ACh activity in the synaptic vesicle suspension was recovered in the 100,000 *g* supernatant. This observation confirms the finding of Whittaker and Sheridan[14] that ACh is released from synaptic vesicles during centrifugation. On the other hand, the extent of release observed in this investigation is in disagreement with the observation of Whittaker *et al.*,[10] who reported that approximately 80 per cent of the ACh in the synaptic vesicle fraction can be pelleted after diluting the suspension 1:1 with distilled water and pelleting at 100,000 *g* for 60 min. The only difference in our methods of pelleting was in the type of rotor used. In the present investigation, a Spinco no. 40 fixed-angle rotor was used, whereas Whittaker *et al.*[10] used the Spinco swing bucket, SW39 rotor. Thus, the increased shear which the vesicles would undergo during precipitation in a fixed-angle rotor may account for the low recovery of ACh in the pellet. Upon injecting aliquots of the vesicle suspension into the assay organ bath at different times during the incubation, it was possible to demonstrate an increase in soluble ACh activity. However, these observations were not always reproducible and were quite difficult to quantitate accurately because the negligible increase in soluble ACh, which occurred early in the incubation period, allowed only a two-point assay. Ultrafiltration was too slow a process to study the relase over the short periods during which a majority of the release occurs at the temperatures used.

Electron microscopy of the 100,000 *g* pellets of the various fractions in the Whittaker procedure shows that the pellet obtained from the W_s fraction (10,000 *g* supernatant of osmotically shocked mitochondrial pellet), which is reported to be equivalent to the M_2 pellet of De Robertis,[15] is heavily contaminated with microsomal membranes. The D (synaptic vesicle) fraction, in comparison, is relatively free of these contaminants. These results are shown in Figs. 1 and 2, which are electron photomicrographs of the W_S and D fractions. Tucek[16] showed that the same is true for the D and M_2 fractions obtained from rat forebrain.

In the series of control experiments, we observed that samples incubated for 20 min in the presence of the neutralized, inactivated enzyme preparation or AChE vehicle displayed no difference from control in amounts or rates of ACh released at 20° or 37°. In the control experiments evaluating the time course of ACh hydrolysis and

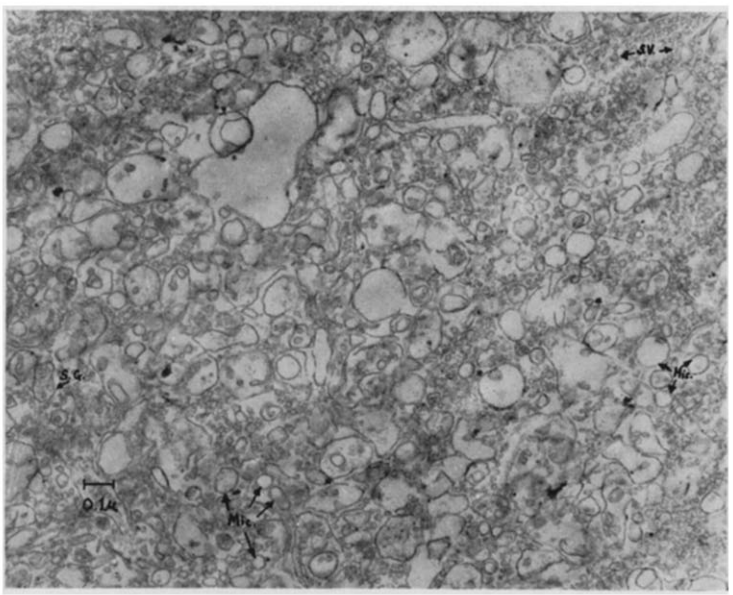

FIG. 1. Electron micrograph of W_5 pellet isolated by centrifugation at 100,000 *g* for 60 min. This micrograph is 1 of 12 sections taken on one W_s pellet. In this micrograph synaptic vesicles (S.V.), synaptosome ghosts (S.G.), and microsomal membranes (Mic.) can be seen.

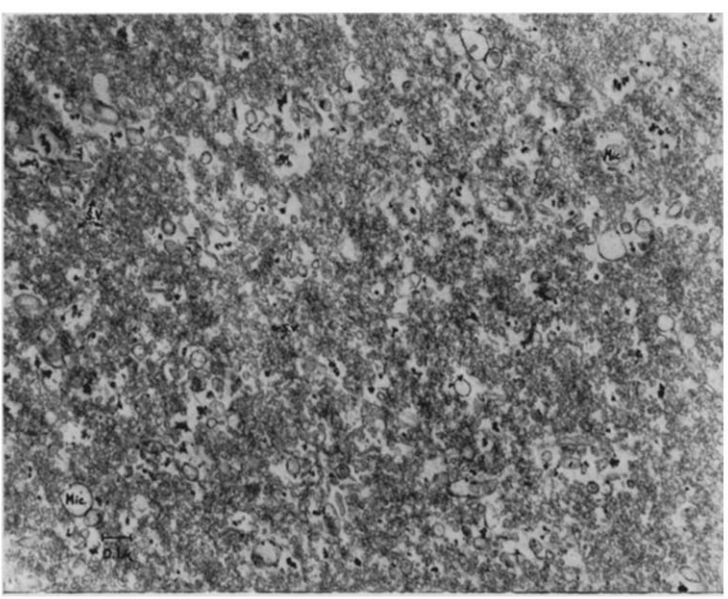

FIG. 2. Electron micrograph of synaptic vesicle pellet isolated by diluting the vesicle suspension 1:1 with disilled water and centrifugation at 100,000 *g* for 60 min. Electron microscopic examination of D pellets from 6 experiments was performed. This micrograph is representative. Only synaptic vesicles (S.V.) and some microsomal membranes (Mic.) were observed. Note the absence of synaptosome ghosts.

enzyme inactivation, the concentration of ACh in the vesicle fraction was 80 nanograms/ml. Complete hydrolysis of ACh occurred within 5 sec. No hydrolysis of ACh occurred when the enzyme was added to a preparation pretreated with sufficient 0·2 N HCl to yield a final pH of 4·0. No loss of ACh activity was observed to occur in the pH 4 samples after 4 hr at room temperature or after storage overnight at −20°. If the pH 4 samples were neutralized, loss of ACh activity occurred on standing at room temperature. This indicates that the inactivation of AChE by decreasing the pH to 4 is partially reversible. Bioassay of ACh standards at pH 4 and pH 7 showed equal activity. The same was found to be the case for the activity in the synaptic vesicle fraction to which no AChE had been added.

From these observations we conclude that: (1) neither AChE nor constituents of the enzyme preparation evokes or enhances the release of ACh from isolated synaptic vesicles; (2) the method we have selected for inactivation of the enzyme, decreasing the pH of the sample to 4 after adding AChE, is effective and occurs at a sufficiently rapid rate to warrant its use; and (3) the ACh activity is stable at pH 4 and this pH does not affect the results of the bioassay. These control experiments validate our initial assumptions that free ACh is rapidly hydrolysed by the added AChE and that the enzyme is inactivated immediately upon attaining a pH of 4·0.

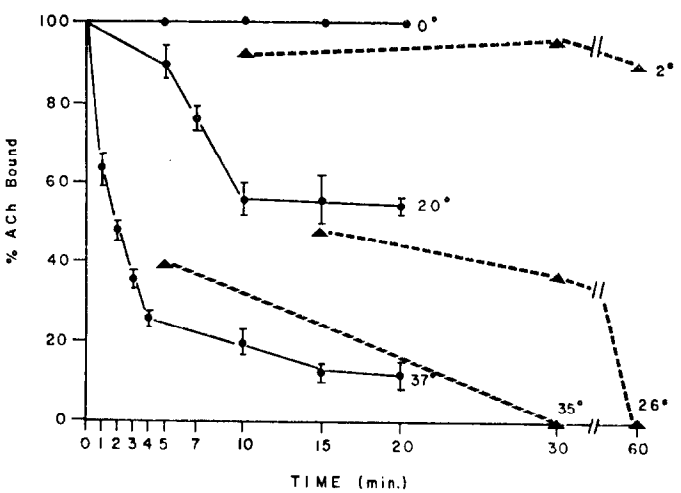

FIG. 3. The effect of temperature on the spontaneous release of ACh from isolated synaptic vesicles suspended in 0·4 M sucrose. Each point (closed circles) and bar represent the mean and standard error for at least 4 experiments. The zero point on the curve represents the amount of ACh initially bound to the vesicles obtained as described in the text and defined as 100 per cent. All other values are expressed as per cent of the zero time value. The average zero time value was 35 ± 3·5 nanograms/ml, which corresponds to a value of 61·3 nanograms/g tissue. The curves at 2° and 26° indicate the results recently obtained by Whittaker and Marchbanks (personal communication), and the curve at 35° depicts the data obtained by Whittaker *et al.*[10]

The effects of temperature on the spontaneous release of ACh are presented in Fig. 3. The possibility exists that the decreases in bound ACh, which occur during incubation at temperatures greater than 0°, are due to enzymic or nonenzymic hydrolysis in or on the vesicles. If such were the case, one would expect to see a progressive decrease in the concentration of ACh in the course of an incubation that is

terminated by addition of HCl only. Such an experiment was performed, and no loss or gain of ACh activity was observed to occur in the course of a 20-min incubation at 20° and 30°. From this observation it is concluded that ACh is released from the synaptic vesicles and not destroyed. Therefore the determination of the amount of bound ACh will allow calculation of the amount of ACh released. Also, this observation established that under the experimental conditions used no net synthesis of ACh occurs.

Also in Fig. 3 are data on the spontaneous release recently obtained by Whittaker and Marchbanks* and that initially reported by Whittaker *et al.*[10] The results of Whittaker and Marchbanks were obtained by allowing the isolated vesicle preparation to stand at various temperatures for various times and then passing the preparation through a Sephadex column in order to exclude the free ester. Bound ACh, in this case, is defined as that coming through the void volume of the column on elution with 0·4 M sucrose. These data are reasonably consistent with our data as well as with the previous data obtained by Whittaker *et al.*[10] The diversity of the two methods implies that the inherent errors of both are different. Thus it may be concluded that both sets of data accurately reflect the release of ACh from isolated synaptic vesicles. The advantage of the AChE method compared to the method of Whittaker and Marchbanks is that it permits one to study the initial rapid phase as well as the latter slow phase of ACh release.

The nature of the release, an initial, rapid and then a later slow phase, suggests that at least two processes are occurring simultaneously. Perhaps this may represent ACh release from the surface as well as from the interior of the synaptic vesicle.

Studies are currently under way on the effect of CPZ and various ions on the spontaneous release of ACh from isolated synaptic vesicles and will be reported in detail later.

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