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Pentobarbitone inhibition of catecholamine secretion

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Summary

1. The perfused isolated cow adrenal gland was used to investigate the effect of barbituric acid, phenobarbitone and pentobarbitone on catecholamine secretion.
2. Pentobarbitone reduced catecholamine secretion induced by a number of drugs which cause exocytosis. The concentration of pentobarbitone which caused a 50% inhibition of catecholamine secretion was for acetylcholine $5{\cdot}6\times10^{-5}$M, for carbachol $6{\cdot}3\times10^{-5}$M, for histamine $1{\cdot}6\times10^{-4}$M, for (+)-amphetamine $4{\cdot}4\times10^{-5}$M and for potassium chloride $1{\cdot}5\times10^{-4}$M. The degree of inhibition by pentobarbitone was not dependent on the concentration of the secretagogue.
3. Pentobarbitone (up to 10^{-3}M) did not inhibit the catecholamine release that was induced by acetyldehyde or by calcium chloride; it inhibited slightly (34%) the catecholamine secretion induced by tyramine.
4. Catecholamine release induced by carbachol was also inhibited by phenobarbitone (50% inhibition at $2{\cdot}8\times10^{-4}$M (n=7)) but was unaffected by barbituric acid.
5. Pentobarbitone had no effect on spontaneous or on (+)-amphetamine- or tyramine-induced release of catecholamines from isolated chromaffin vesicles of cow adrenal medulla.
6. It is concluded that pentobarbitone inhibits catecholamine release by preventing a configurational change in the structure of the membrane of the chromaffin cell which is a necessary link between receptor activation and catecholamine release.

Introduction

Hypnotic derivatives of barbituric acid produce depression in a number of biological systems, including nerve axons (Schoepfle, 1957; Blaustein, 1968), ganglia (Exley, 1954; Sato, Austin & Yai, 1967), the neuromuscular junction (Thesleff, 1956; Galindo, 1971), and the monosynaptic spinal reflex arc (Løyning, Oshima & Yokota, 1964). The cellular mechanism or mechanisms responsible for this characteristic effect are not known, although several hypotheses have been formulated to explain their depressant action. Inhibition by barbiturates of both presynaptic events (Løyning *et al.*, 1964; Weakly, 1969; Galindo, 1971) and postsynaptic events (Somjen & Gill, 1963; Galindo, 1969) have been postulated. However, whether barbiturates act presynaptically, postsynaptically or both, it

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is highly likely that their effects on the brain involve alteration of synaptic transmission. The purpose of the work reported here was to study further the effects of barbiturates on one particular aspect of synaptic transmission, stimulus-release coupling. The adrenal medulla was used as a model in which to study this phenomenon since much is known about the biochemistry and cellular dynamics of catecholamine secretion in response to stimulation of the cholinergic receptors of chromaffin cells.

Methods

Release of chromaffin vesicle constituents from the perfused adrenal

Cow adrenal glands weighing between 12 and 20 g were obtained approximately 15 min after the animals had been killed and were kept in ice for 30 to 60 min until perfusion was begun. The glands were perfused for 60 to 90 min before stimulation. Catecholamine (CA) release was stimulated as previously described (Schneider, Smith & Winkler, 1967). Solutions of the stimulating drugs were infused for 1 min and 20 s into the perfusion medium at a rate of either 1·5 ml/min or 0·8 ml/min with a Harvard infusion pump at a point immediately before the fluid entered the tissue. The perfusate flow was maintained between 10 and 11·5 ml/minute. The drug concentrations indicated for the perfusion experiments represent the concentrations in the fluid perfusing the gland. The drugs used to stimulate catecholamine release (secretagogues) were used in concentrations that provided responses between 60 and 90% of their maximum responses.

The perfusates (1·5 ml) were analysed for total catecholamines by the colourimetric method of von Euler & Hamberg (1949) using citrate-phosphate buffer at pH 6·0 (McIlvaine, 1921). Samples of the perfusates collected for one min 60 to 90 min after beginning perfusion contained between 0·2 and 0·7 μmol of catecholamines. The proteins in perfusates were precipitated with trichloracetic acid (final concentration, 5% v/v) and were assayed by the method of Goa (1953). Bovine serum albumin was used as a standard. The protein content of perfusates collected for one min 60 to 90 min after beginning perfusion was between 0·2 and 1·4 mg. The effect of a secretagogue was expressed either as the absolute increase in catecholamines released during the stimulation period [(μmol CA in stimulation period)−(μmol CA in prestimulation period)], or as the increase in percent above the basal secretion [[(μmol CA in stimulation period−(μmol CA in prestimulation period)×100]/(μmol CA in prestimulation period)]. The stimulation period consisted of the time during which the drug was infused (1 min and 20 s) and the 4 min and 40 s immediately following; the prestimulation period consisted of an equal time period (6 min) immediately before the stimulation period. Increase in protein release during stimulation was calculated similarly.

Calculation of drug-induced inhibition of catecholamine secretion

The ability of a drug to reduce or enhance the catecholamine or protein release in response to a secretagogue was assessed by comparing the responses during exposure to the drug with the responses obtained before and after the exposure to the drug. The following time sequence was used in these experiments: (a) perfusion with normal Tyrode solution for 90 min, (b) a series of 6 min

alternating control periods and 6 min stimulation periods (1 min 20 s stimulation +4 min 40 s post-stimulation), (c) perfusion with Tyrode solution containing the drug being tested for inhibition for 20 min, (d) second series of alternating control (6 min) and (e) stimulation periods (6 min), while perfusion with 'inhibitor' in Tyrode solution was continued, (f) perfusion with normal Tyrode for 30 min, (g) a third series of control and stimulation periods of 6 min each. The first stimulation for each gland was not used in the calculations since the initial release of catecholamines was usually considerably higher than subsequent responses. If more than one stimulation period occurred during perfusion with either normal or drug-containing Tyrode solution, 20 min separated one stimulation period from the next control collection period. The equation for the straight line fitting the time (in min) and catecholamine release responses was calculated from the data obtained during perfusion with normal perfusion fluid and was used to predict the responses that would have been obtained at the time of stimulation during perfusion with drug-containing perfusion fluid. The predicted response was compared with the response obtained, and inhibition (or potentiation) was calculated by the equation:

$$\text{Inhibition} = \frac{(\text{predicted response}) - (\text{observed response}) \times 100}{(\text{predicted response})}$$

The values for inhibition obtained either with responses expressed as the increase in μmol or as the precent increase were similar. Primarily values expressed as increases in μmol are used in this paper. This method is illustrated in the lower portion of Fig. 1 for stimulation with carbachol and potassium chloride, and with phenobarbitone as the inhibitory agent.

Isolated chromaffin vesicles

Incubation studies were carried out on 'large vesicle fractions' prepared from medullary tissue of bovine adrenal glands (Schneider, 1971a) and incubated for 5 min at 37° C as previously described (Schneider, 1971b). The incubation mixture was then cooled to 4° C and centrifuged at 48,000 *g* for 30 minutes. Portions of the resulting supernatant were assayed for catecholamines and for protein. The large vesicle fraction incubation mixture contained a total of 2 to 3 μmol of catecholamines in each incubation sample.

Statistical analyses were performed with the Olivetti Underwood Programma 101 desk computer with programmes described in the Olivetti Underwood Statistical Analysis handbook (1968).

Drugs

Dextroamphetamine sulphate (+-amphetamine) was supplied by L. S. Jaffe, Smith, Kline & French Laboratories, tranylcypromine sulphate was supplied by L. B. Luongo, Smith, Kline & French Laboratories, and pentobarbitone sodium was supplied by A. O. Willy, Abbott Laboratories. Other drugs were obtained through commercial sources.

Results

Effect of pentobarbitone on carbachol-induced exocytosis

Stimulation of the perfused isolated cow adrenal gland with carbachol caused

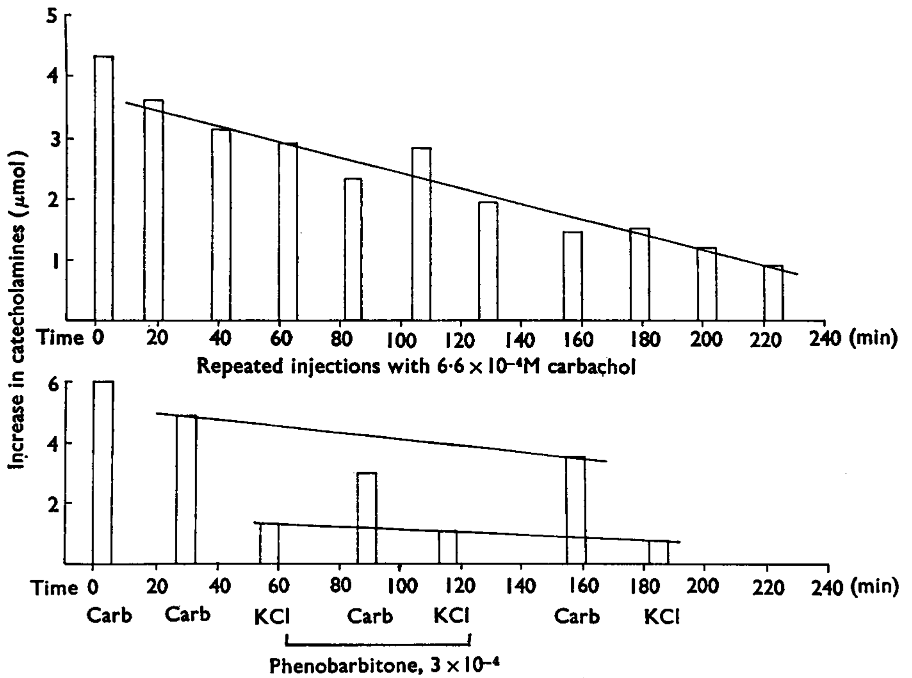

FIG. 1. Catecholamine release from the isolated cow adrenal gland and its inhibition by phenobarbitone. The glands were perfused for 90 min before the first stimulation. Collection periods were 6 min; each stimulation period consisted of 1 min and 20 s of drug infusion followed by 4 min and 40 s of normal Tyrode. The bars represent μmol of catecholamines released during the stimulation period minus μmol of catecholamines released in the preceding 6-min perstimulation period. Lower panel: Carb: stimulation with $2{\cdot}6\times10^{-3}$M carbachol; KCl: stimulation with 7×10^{-2}M potassium chloride. See Methods section for experimental details.

the release of the soluble contents of chromaffin vesicles, including catecholamines, adenine nucleotides and protein. Figure 1, upper panel, shows the release of catecholamines upon repeated infusions of $6{\cdot}6\times10^{-4}$M carbachol for 1 min 20 seconds. The responses, whether calculated as μmol of catecholamines or as the percent increase above basal release, showed a gradual decline in magnitude with repeated stimulation. The effect of an antagonist on the catecholamine secretion induced by a secretagogue can be assessed as demonstrated in the lower portion of Figure 1. Responses to carbachol and potassium chloride were measured before and during exposure of the gland to an antagonist, in this case, phenobarbitone. The predicted values for the times corresponding to stimulation during exposure to the antagonist are compared with the actual values obtained. This method can be used with the responses expressed either as μmol of catecholamines released above basal levels as in Fig. 1, or as the percent increase above basal levels. In this example phenobarbitone did not alter the response to potassium chloride, although at higher concentrations inhibition of the response to potassium chloride was observed.

The effect of the presence of pentobarbitone on basal catecholamine release was quite variable at concentrations below 10^{-4}M; 20 min after exposure to 10^{-4}M and 10^{-3}M pentobarbitone decreases in the spontaneous release of CA of $22\pm9\%$ ($n=5$; $P<0{\cdot}05$) and $26\pm9\%$ ($n=4$; $P<0{\cdot}05$), respectively, were observed. The effect of pentobarbitone in concentrations between 3×10^{-6} and 10^{-3}M on

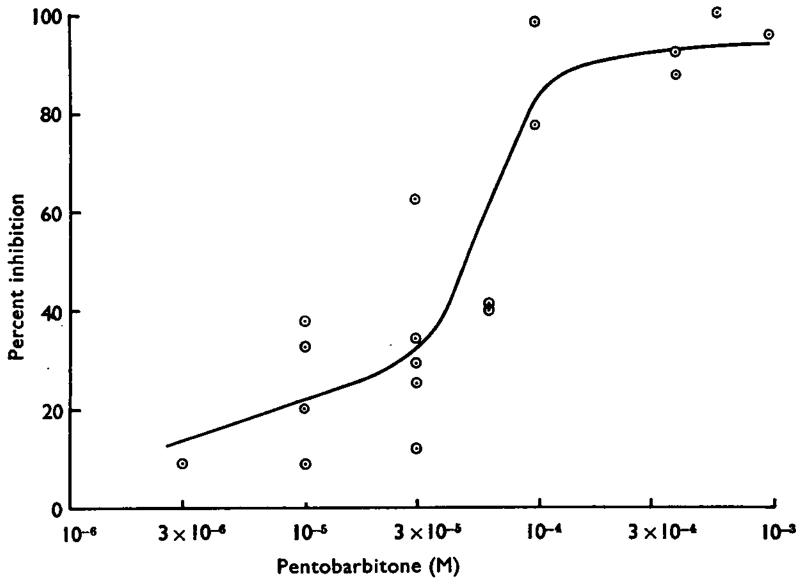

FIG. 2. Inhibition by sodium pentobarbitone of carbachol-induced catecholamine secretion. Catecholamine secretion in response to exposure to carbachol ($2{\cdot}6\times10^{-3}$M) is expressed as the μmolar increase above the basal level of release.

the release of catecholamines induced by carbachol ($2{\cdot}6\times10^{-3}$M) is shown in Figure 2. At concentrations of 3×10^{-5}M and below, the catecholamine release is inhibited to a variable degree. For pentobarbitone concentrations between 10^{-5} and 10^{-3}M a positive correlation [$r=+0{\cdot}64$ ($n=15$, $P<0{\cdot}01$)] existed between the inhibition of the release of catecholamines and the concentration of pentobarbitone when catecholamine release was expressed as μmol above the basal release. The concentration of pentobarbitone that produced a 50% inhibition of catecholamine release was calculated from the equation of the straight line through the linear portion of the inhibition concentration curve. When catecholamine release is expressed as percent release, 50% inhibition occurred at $6{\cdot}2\times10^{-5}$M pentobarbitone; 50% inhibition was at $6{\cdot}3\times10^{-5}$M when responses were plotted as the change in μmol released. A 50% inhibition of the carbachol ($2{\cdot}6\times10^{-3}$M)-induced protein release (expressed as mg increase above the basal level) was also observed in the presence of $6{\cdot}3\times10^{-5}$M pentobarbitone. Inhibition of catecholamine and protein release was readily reversible upon perfusion with pentobarbitone-free perfusion fluid.

An inhibition by pentobarbitone of the catecholamine secretion stimulated by

TABLE 1. *Pentobarbitone-induced inhibition of catecholamine secretion by the perfused adrenal gland*

Secretagogue		Concentration of pentobarbitone (M) giving a 50% inhibition of catecholamine release
Acetylcholine	($1{\cdot}3\times10^{-3}$M; $n=27$)	$5{\cdot}6\times10^{-5}$
Carbachol	($2{\cdot}6\times10^{-3}$M; $n=11$)	$6{\cdot}3\times10^{-5}$
Histamine	($1{\cdot}1\times10^{-3}$M; $n=11$)	$1{\cdot}6\times10^{-4}$
Potassium chloride	($7{\cdot}0\times10^{-2}$M; $n=9$)	$1{\cdot}5\times10^{-4}$
(+)−Amphetamine	($7{\cdot}1\times10^{-4}$M; $n=16$)	$4{\cdot}4\times10^{-5}$

The figures in the parentheses after each drug represent its concentration and the number (n) of experiments. Catecholamine secretion in response to stimulation with the secretagogues was expressed as the absolute increase in μmol.

other secretagogues is shown in Table 1. The values for 50% inhibition range between $6{\cdot}2\times10^{-5}$M pentobarbitone for acetylcholine and $1{\cdot}6\times10^{-4}$M pentobarbitone for histamine, and were similar when calculated with the catecholamine release expressed either as μmol or as percent increase above prestimulation levels. Pentobarbitone in molar concentrations of 3×10^{-5} ($n=2$), 3×10^{-4} ($n=2$) and 10^{-3} ($n=2$) did not inhibit catecholamine release induced by $3{\cdot}3\times10^{-2}$M acetaldehyde. Pentobarbitone (3×10^{-4}M) inhibited tyramine ($7{\cdot}1\times10^{-3}$M)-induced catecholamine release $34{\cdot}2\pm6{\cdot}4$% ($n=5$; $P<0{\cdot}01$). Pentobarbitone (3×10^{-4}M) inhibited the CA release induced by (+)-amphetamine ($7{\cdot}1\times10^{-3}$M) by $80{\cdot}0\pm8{\cdot}3$% ($n=6$; $P<0{\cdot}01$).

Since the action of each of the secretagogues that is antagonized by pentobarbitone (with the possible exception of tyramine) is dependent upon the presence of calcium in the perfusion fluid, the ability of pentobarbitone to block calcium-induced catecholamine secretion was examined. Catecholamine release in calcium-free Tyrode solution in response to calcium (19 mM) was 105 ± 12% ($n=41$) above basal levels; protein release was 44 ± 5% ($n=41$) above resting levels. There was no inhibition of calcium-induced catecholamine secretion in the presence of 3×10^{-5} ($n=8$), 3×10^{-4} ($n=14$) or 10^{+3}M ($n=10$) pentobarbitone. Protein secretion by Ca^{++} was decreased by 23% ±8% ($n=5$; $P<0{\cdot}05$), in the presence of 10^{-3}M pentobarbitone.

Effects of other barbituric acid derivatives on catecholamine release

Phenobarbitone also reduced catecholamine secretion by carbachol, although the concentration required for a 50% inhibition was higher than with pentobarbitone; 50% inhibition was obtained at $1{\cdot}8\times10^{-4}$M ($n=7$) when catecholamine secretion was expressed as percent increase above prestimulation level and $2{\cdot}8\times10^{-4}$M ($n=7$) when catecholamine secretion was expressed as the increase in μmol above prestimulation levels. Barbituric acid between 3×10^{-6} and 3×10^{-3}M did not inhibit catecholamine release induced by carbachol ($2{\cdot}6\times10^{-3}$M), and at 3×10^{-4}M ($n=9$) did not inhibit catecholamine secretion stimulated by exposure of the gland to 19 mM $CaCl_2$ during perfusion with calcium-free perfusion fluid.

Type of inhibition produced by pentobarbitone

If pentobarbitone produced inhibition of the carbachol-induced catecholamine release by blocking acetylcholine receptors the inhibition would be of the reversible or surmountable type since inhibition is readily reversible upon perfusion with pentobarbitone-free fluid. The type of inhibition was tested by stimulating the

TABLE 2. *Percent inhibition by pentobarbitone of the carbachol-induced release of catecholamines by the perfused adrenal gland*

Pentobarbitone	Carbachol				
	$8{\cdot}6\times19^{-5}$M	$2{\cdot}6\times10^{-4}$M	$8{\cdot}6\times10^{-4}$M	$2{\cdot}6\times10^{-3}$M	$8{\cdot}6\times10^{-3}$M
3×10^{-5}M	21·0%±16·5 (6)	27·5%±16·5 (4)	25·0%± 7·3 (5)	22·1%± 7·3 (9)	40·0%±22·9 (3)
6×10^{-5}M	32·7%± 6·9 (4)	53·7%± 7·2 (3)	36·5%±11·5 (3)	37·9%± 3·6 (5)	26·7%±15·0 (4)

The figures represent mean percent inhibition and standard errors. Catecholamine release is expressed as the increase in μmol. In parentheses: number of experiments.

gland with varying concentrations of a secretagogue in the presence of a constant concentration of pentobarbitone. For 2 concentrations of pentobarbitone there is no relation between the inhibitor concentration and inhibition of catecholamine release over the range of $8{\cdot}6\times10^{-5}$ to $8{\cdot}6\times10^{-3}$M carbachol (Table 2). For 3×10^{-5}M pentobarbitone the correlation coefficient was 0·1032 ($P<0{\cdot}05$) and for 6×10^{-5}M was 0·3848 ($P<0{\cdot}05$). These results indicate that the inhibition was of the unsurmountable type.

Effect of pentobarbitone on cow adrenal medulla chromaffin vesicles

Pentobarbitone at 3×10^{-6}, 3×10^{-5}, 3×10^{-4} and 3×10^{-3}M had no effect on either spontaneous or (+)-amphetamine (3×10^{-3}M)—or tyramine (3×10^{-3}M)-induced release of catecholamines from chromaffin vesicles incubated in Tyrode solution for 5 min at 37° C. (+)-Amphetamine (3×10^{-3}M) caused a 255 ± 41% ($n=5$; $P<0{\cdot}01$) increase in catecholamine release and tyramine (3×10^{-3}M) caused a 138 ± 17% ($n=6$; $P<0{\cdot}01$) increase.

Discussion

The results presented here show that pentobarbitone reduces catecholamine secretion from the adrenal medulla in response to stimulation by certain agents that induce exocytosis. This finding is in agreement with previous work showing

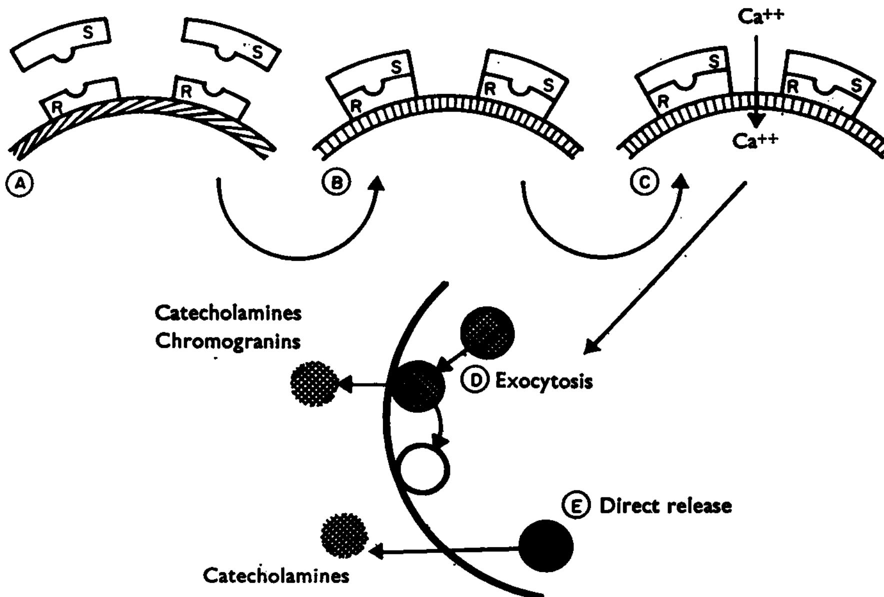

FIG. 3. Events in catecholamine release from chromaffin cells of the adrenal medulla. Step A: stimulation of the cells by secretagogues, e.g., acetylcholine, carbachol or histamine (S), which interact with a specific receptor (R). Step B: change in membrane structure that occurs as a result of receptor activation. The alteration in membrane configuration can also be produced by depolarization with potassium chloride. Step C: increase in permeability of the membrane to calcium, the result of which is an increase in intracellular calcium concentration. Step D: the result of the increase in intracellular calcium is exocytosis, the process by which all the soluble contents of chromaffin vesicles are extruded across the membrane into the extracellular space. There are also drugs that release catecholamines by a direct interaction with the chromaffin vesicles, such as acetaldehyde. This release mechanism is shown as E.

barbiturate depression of excitable tissues (see Introduction). Furthermore, barbituric acid derivatives reduce catecholamine release from adrenal glands *in situ* (Montagu, 1955 ; Weil-Malherbe, 1955 ; Walker, Zileli, Reutter, Shoemaker & Moore, 1959).

The cellular events occurring during exocytotic release of catecholamines from chromaffin cells of the adrenal medulla are illustrated in Figure. 3. Stimulus-secretion coupling involves 1) stimuluation of the cell by an appropriate agonist, which leads to, 2) a structural change in the cell membrane, which allows 3) an increase in intracellular free calcium, and 4) association of the catecholamine-containing chromaffin vesicles with the cell membrane, followed by 5) efflux of the soluble constituents of the chromaffin vesicle. Barbiturates could interfere with catecholamine release by 1) acting as receptor blocking agents, 2) stabilization of the membrane in a way that prevents a necessary configurational alteration, and 3) blockade of calcium influx or mobilization.

Since pentobarbitone blocks not only the effect of substances which stimulate catecholamine release by stimulation of acetylcholine receptors but also the effect of histamine and potassium chloride, it is unlikely that receptor blockade is responsible for its inhibitory effect. This conclusion is strengthened by the finding that inhibition of the response to carbachol is of an unsurmountable type. Inhibition by pentobarbitone of the calcium flux that normally occurs as a result of stimulation is unlikely since calcium is capable of inducing catecholamine release in the presence of pentobarbitone in concentrations that inhibit acetylcholine- or carbachol-stimulated release.

An effect of pentobarbitone on chromaffin vesicles is unlikely as an explanation for the inhibitory effect of pentobarbitone since pentobarbitone did not affect the loss of catecholamines from isolated chromaffin vesicles. It is improbable that the barbiturate reduces migration of chromaffin vesicle components across the chromaffin cell membrane since neither calcium-induced exocytosis nor acetaldehyde-induced catecholamine secretion was affected.

Although barbiturates can block energy production, it is unlikely that at the concentrations used in this study pentobarbitone is exerting its inhibitory action by this mechanism. Concentrations of barbiturates required to block energy metabolism are considerably higher than those causing catecholamine inhibition in this study, generally above 10^{-3}M (see Mahler & Cordes, 1966).

It is concluded that pentobarbitone inhibits catecholamine release in response to drugs which stimulate exocytosis by preventing the stimulus-induced alteration in the cell membrane. An action of this nature is consistent with the blockade of the stimulatory effect of acetylcholine, carbachol, histamine and potassium chloride and a lack of an effect on the actions of calcium and acetaldehyde. Acetaldehyde releases catecholamines by a process other than exocytosis, possibly by direct interaction with the chromaffin vesicle (Schneider, 1971a). The action of acetaldehyde is illustrated by step E in Figure 3. The ability of pentobarbitone to block the action of (+)-amphetamine is of interest in view of recent findings that (+)-amphetamine stimulates exocytosis in the adrenal medulla (Schneider, 1971b). The mechanism of the 34% reduction in tyramine-induced catecholamine release is at the present time not known.

It is also possible that inhibitory barbiturates combine with the cell membrane

in a way that prevents a critical configurational alteration (step B, Fig. 3) which normally occurs in response to receptor activation or to an ionic imbalance, such as that obtained by stimulation with potassium chloride. Recent electrophysiological experiments by Clark & Strickholm (1971) suggest that nerve excitation involves a change in the conformation of membrane-bound protein. It may be that various drugs which stabilize excitable membranes prevent changes of this type, and barbiturates may exert at least a portion of their effect in this manner. This is in agreement with the suggestion made over 20 years ago by Eccles (1946) that barbiturates act by increasing the stability of the nerve cell membrane to electrical changes.

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