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THE RELEASE OF CATECHOL AMINES FROM ISOLATED CHROMAFFIN GRANULES

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Release of catechol amines from the chromaffin granules of the bovine suprarenal medulla has been studied. Aliphatic monoamines and diamines released catechol amines from chromaffin granules under conditions similar to those under which they are known to promote the release of histamine from suspensions of granules. Carbachol and histamine did not release catechol amines from granules.

Recent work has shown that the pharmacologically active amines are stored in cytoplasmic granules. However, little is known about the mechanism of release from these granules. In his study of the intracellular localization of histamine, Hagen (1954) found that the amine present in a fresh suspension of dog liver granules in isotonic sucrose did not exert its effect upon the blood pressure of the decerebrate cat, but that it was immediately and fully active upon treatment of the suspension with a histamine releaser, *n*-octylamine. Other substances have since also been found to release histamine from a suspension of granules (Mongar and Schild, 1954, 1956; Grossberg and Garcia-Arocha, 1954).

Undenatured chromaffin granules, when injected into the spinal cat, do not immediately exert the full pressor effect which would correspond to their catecol amine content (Blaschko, Hagen, and Welch, 1955). Some time ago, Feldberg (1940) had shown that lysolecithin released adrenaline from "cell debris," and Hillarp and Nilson (1954) have since found that not only lysolecithin but a number of surface-active agents release catechol amines from suspensions of chromaffin granules into the surrounding fluid.

In the present work, the action of a number of compounds, known to be releasers of histamine, was studied. Observations were also made on the action of substances known to release catechol amines from intact chromaffin cells *in situ*.

These results were communicated to the British Pharmacological Society in January, 1956.

METHODS

Preparation of the Large Granule Fraction.—Ox suprarenal glands were collected on ice from the slaughter-house; usually four glands were dissected and the chopped medullae, about 7 to 10 g., were dispersed in a glass homogenizer in 0.3 M-sucrose. The final homogenate was brought to a volume of 50 ml. and then centrifuged for 10 min. at 2,000 rev./min. (950 *g*) in a refrigerated MSE centrifuge. The supernatant fluid, usually 40 to 43 ml., was collected; the sediment, which contained coarse material, red cells, and nuclei, was discarded. The supernatant fluid was again centrifuged, this time in the high-speed attachment of the MSE centrifuge at 2,500 × 4.75 rev./min. (11,000 *g*) for 20 min. At the end of the run the supernatant fluid was decanted and discarded. The inner walls of the centrifuge tubes were wiped dry with tissue-paper and the sediments resuspended by adding 0.3 M-sucrose, so as to bring the suspension up to the original volume. The sediments were thoroughly stirred and the suspension obtained was collected and shaken. This suspension was again centrifuged, in order to reduce the amounts of amine in free solution, this time at 2,800 × 4.75 rev./min. (14,000 *g*) for 20 min. The supernatant fluid was discarded and the sediment resuspended in 0.3 M-sucrose as before. The final volume of the suspension was chosen so that 1 g. of medulla corresponded to 2 ml. of suspension. In the following, this suspension will be referred to as the "large-granule fraction"; it is known that this fraction, which contains the greater part of the catechol amines, is not homogeneous, but can be further subdivided into "top" and "bottom" layers (Blaschko, Hagen, and Welch, 1955).

All centrifugations were carried out at 0° C., and during the preparation all manipulations were carried out as near 0° C. as possible.

Incubation.—The large-granule fraction was incubated under various conditions in order to study the release of catechol amines. Centrifuge tubes of 7 ml. capacity were used; the volume of the final suspension was usually 5.0 ml. This volume included 1.0 ml. of the large-granule fraction and 1.0 ml. of phosphate buffer of *p*H 7.0 (Clark, 1928). (A buffer of appro-

priate *p*H was used in those experiments in which the effect of *p*H was studied.) The total volume also included the substance to be tested as a releaser, dissolved or suspended in 0.3 M-sucrose; the final volume was brought up to 5.0 ml. with 0.3 M-sucrose. Where necessary, the substances to be tested were neutralized.

Unless stated otherwise, incubation was for 30 min. in a water-bath at 37.5° C.; the tubes were agitated during incubation. They were at once transferred to an ice-water bath, and then centrifuged at a speed of 3,000 to 4,000 × 4.75 rev./min. (16,000 to 28,000 *g*) for 30 min. The supernatant fluids, which were clear, were collected for the determination of their catechol amine content.

Determination of Catechol Amines.—For this purpose, 1 ml. of the suspension was used; 1.0 ml. of N-HCl was added and the mixture was frozen. It was stored in the refrigerator at −12° C. After thawing, 0.5 ml. of the mixture was added dropwise to 4.5 ml. of cold ethanol; this was allowed to stand in the cold for ½ to 1 hr., and was then centrifuged at 450 *g* for 10 min.; the supernatant fluid from this centrifugation was used for the determination.

The catechol amines released were determined directly in the supernatant fluids after high-speed centrifugation. The method was essentially that of v. Euler and Hamberg (1949), in which the intensity of the colour produced when catechol amines are oxidized by adding iodine is measured. The observation that at *p*H 6.0 adrenaline and noradrenaline contribute equally to the development of colour was confirmed; the determinations were therefore carried out at *p*H 6.0.

After addition of the iodine the samples were allowed to stand for 3 min.; sodium thiosulphate was then added to remove the excess iodine, and the colour intensity was read immediately at 529 mμ in a Unicam spectrophotometer.

Substances Used.—The aliphatic diamines were kindly given by Dr. E. J. Zaimis, and Dr. R. Charlier, of Labaz Laboratories, Brussels, supplied compound L–1935; the other substances were obtained from commercial sources.

RESULTS

The Effect of Temperature upon Release

When a suspension of the large-granule fraction was incubated, the amount of amine released depended upon the temperature at which the incubation was carried out. In the experiment shown in Fig. 1, incubation was at 0°, 25° and at 37.5° C.; samples were removed at different intervals and centrifuged. Fig. 1 shows the amount of catechol amine found in the supernatant fluids. In this and subsequent experiments, about 10% of catechol amines was present in the supernatant fluid at zero time of incubation. This was probably amine not completely removed when the granules were washed.

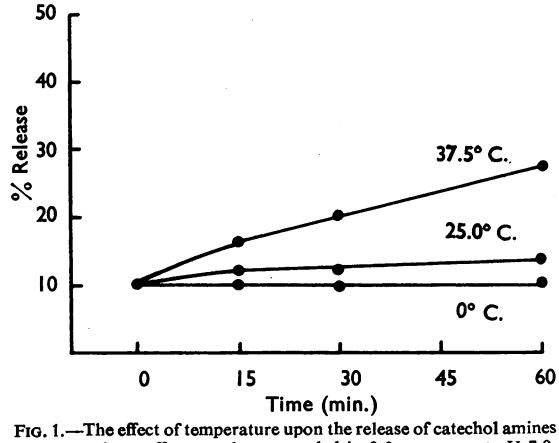

FIG. 1.—The effect of temperature upon the release of catechol amines from chromaffin granules suspended in 0.3M-sucrose at *p*H 7.0. Abscissa: time in min. Ordinate: % of catechol amines released. Temperatures: 0°, 25°, and 37.5° C.

Upon incubation at 0° C., no release of amines occurred and at 25° C. the amounts released were small. At 37.5° C., release was more marked: of the amines present in the granules at the beginning of the incubation (90% of the total amine present in the sample) 18%, or one-fifth, were released during one hour at 37.5° C.

Homogenates in Saline Media

A few comparisons were made of amine release when the medullary tissue was homogenized in the sucrose medium or in a saline medium. In these experiments, the chopped medullary tissue was divided into two portions, one of which was homogenized in 0.3 M-sucrose, the other in the saline medium. Two different saline media were tested in different experiments: one was a mixture of nine parts of 0.15M-NaCl and one part of 0.067M-sodium phosphate buffer of *p*H 7.4. The other medium contained nine parts of 0.15M-KCl and one part of 0.067M-potassium phosphate buffer of *p*H 7.4. Four experiments were carried out, two with the sodium chloride and two with the potassium chloride medium respectively. There was no evidence that homogenizing in an electrolyte medium favoured release. However, there was some evidence that, upon incubation of a large-granule fraction, release was favoured in a saline medium.

The Action of Releasers

Aliphatic Monoamines.—The substances tested included the straight-chain aliphatic monoamines of the series $CH_3(CH_2)_{n-1}NH_2$. All the members of the series from $n=3$ to $n=10$ were available, as well as the amines with 12 and 18 carbon atoms.

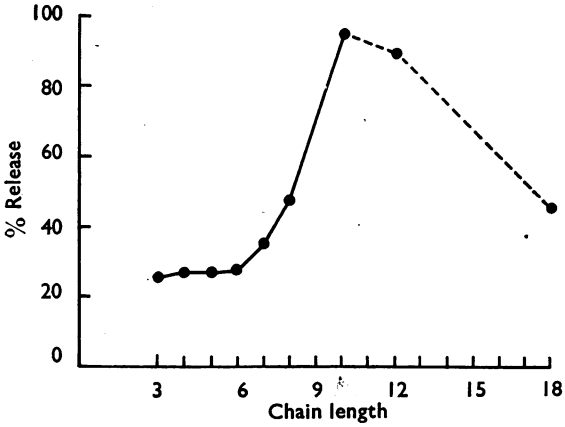

FIG. 2.—Release of catechol amines by aliphatic monoamines of the series $CH_3(CH_2)_{n-1}NH_2$. Abscissa: number of carbon atoms. Ordinate: % release. Incubation at 37.5° C. and *p*H 7.0 in 0.3M-sucrose for 30 min. Amine concentration: 0.97×10^{-3}M.

The results of experiments, in which the amines were tested in a concentration of 0.97×10^{-3}M, are shown in Fig. 2. With increasing length of the polymethylene chain, there occurred an increase in the amount of catechol amines released; the release was maximal for *n*-decylamine. Doubling the concentration of the releasing agents increased the amounts of catechol amines released.

Octadecylamine ($n=18$) did not give a clear aqueous solution in the concentrations used. On incubation with both octadecylamine and dodecylamine the granules appeared to coagulate with the formation of a flocculent precipitate.

Aliphatic Diamines.—Of the series of straight-chain diamines, $NH_2(CH_2)_nNH_2$, the members from $n=3$ to $n=15$ were tested in a concentration of 0.52×10^{-3}M and 1.04×10^{-3}M.

Release of catechol amines by the aliphatic diamines was less than that observed in the presence of the corresponding monoamines of the same chain length in a similar concentration. The relation between chain length and release in the diamine series is shown in Fig. 3; release increased with increasing length of the polymethylene chain, up to the member with 15 methylene groups.

Other Compounds.—Two surface-active substances were tested: saponin and sodium taurocholate. With sodium taurocholate a concentration of 1 mg./ml. was required to release 50% of the catechol amines into the supernatant fluid. For saponin, the corresponding concentration was about 0.2 mg./ml.

The compound L–1935 was tested in a concentration range of 0.1 to 1.0 mg./ml. About 50% of the catechol amines were found in the supernatant fluid after incubation with 0.7 mg./ml. of L–1935. This substance has a brown colour, and it was noted that upon centrifugation the supernatant fluid had become colourless, whereas the sedimented granules were dark brown, in contrast to their normal tan colour; this indicated that the compound L–1935 had been absorbed by the material of the granules.

Carbachol and histamine were tested, as these two substances are known to release adrenaline from the adrenal medulla *in situ*. No release of amines was observed with concentrations from 0.1 to 1.0 mg./ml.

Propamidine was tested in one experiment at a concentration of 0.5 mg./ml.; it was almost without releasing activity.

*p*H and Release of Catechol Amines

Effect upon Spontaneous Release.—When incubation was carried out at 20° C. there was no marked difference in the release of amines between *p*H 5.0 and 8.0. At 30° C., however, there was a significantly higher release at *p*H 5.0, whereas there was little difference at *p*H 6.0, 7.0 and 8.0. This latter result is in general agreement with findings by Hillarp and Nilson (1954).

Effect upon Chemical Release.—Some of the releasing agents tested were incubated with the large-granule fraction at different *p*H and the release of catechol amines was determined. In these experiments the McIlvaine buffer of *p*H 7.0

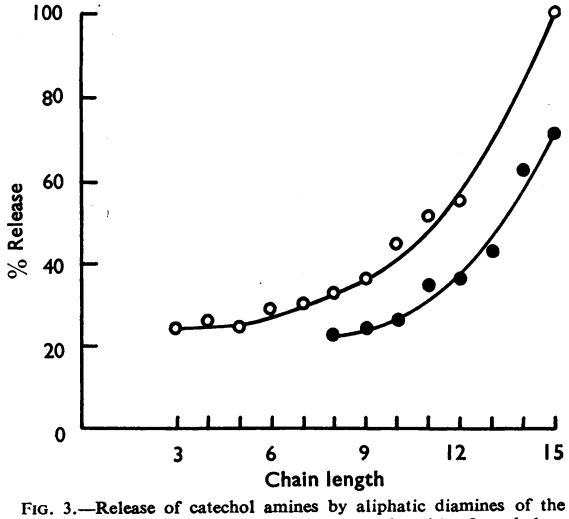

FIG. 3.—Release of catechol amines by aliphatic diamines of the series $NH_2(CH_2)_nNH_2$. Abscissa: number (*n*) of methylene groups. Ordinate: % release. Incubation as in Fig. 2. ●——● Amine concentration 0.52×10^{-3}M; ○——○ Amine concentration 1.04×10^{-3}M.

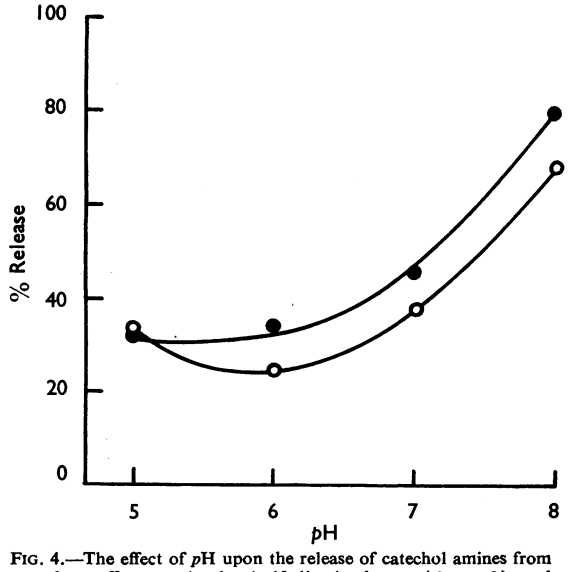

FIG. 4.—The effect of *p*H upon the release of catechol amines from chromaffin granules by 1: 10-diaminodecane (○——○) and L–1935 (●——●). Abscissa: *p*H. Ordinate: % release. Incubation at 37.5° C. for 30 min.

was replaced by the same amount of McIlvaine buffer of different *p*H (Clark, 1928).

In one experiment the large-granule fraction was incubated with *n*-octylamine in a concentration of 1 mg./ml. at 20° C. for 15 min. Upon centrifugation after incubation, the release of catechol amines was greater as the *p*H increased. Thus the amounts at *p*H 5.0, 7.0, and 8.0 were 23%, 71%, and 87% respectively.

In the experiments shown in Fig. 4, incubation was carried out at 37.5° C. and for 30 min.; the *p*H was 5.0, 6.0, 7.0, and 8.0. The two substances tested were 1 : 10-diaminodecane (0.5 mg./ml.) and L–1935 (0.5 mg./ml.). Fig. 4 shows that with diaminodecane there was a minimum of release at *p*H 6.0; at *p*H 5.0, the amount of catechol amines released was slightly greater, and at *p*H 7.0 and 8.0 there was a marked increase in the amounts released. With L–1935, the release at *p*H 5.0 was about the same as at *p*H 6; but here, too, the amounts released at *p*H 7.0 and 8.0 were greater.

DISCUSSION

The release of catechol amines from the intact adrenal medullary cell is usually mediated by nerve stimulation. The secretory nerve supply to the gland is a cholinergic one. That carbachol releases adrenaline from the adrenal medulla in the intact animal was demonstrated by Feldberg (1932). In the experiments here reported carbachol proved ineffective as a releaser of catechol amines from chromaffin granules. Another substance which mobilizes the amines from the intact gland *in situ* is histamine (Burn and Dale, 1926); histamine also was inactive as a releaser. These observations suggest that the action of choline esters and of histamine upon the chromaffin cell is an action upon the cell membrane. The nature of this effect upon the cell membrane is not known; possibly it consists in an increased permeability of the membrane, which allows amines present in the cytoplasmic sap to leave the cell and to enter the surrounding tissue fluids and blood. There seems to be no direct effect of these substances upon the storage granules.

A number of other substances were found which release catechol amines from isolated granules. Some of these substances, such as the aliphatic mono- and di-amines, appear to be as active releasers of catechol amines as they are of histamine. That the mechanism of catechol amine release is similar to the release of histamine is further supported by the observation that compound L–1935, which also releases catechol amines, is also known to be an active histamine releaser (Feldberg and Lecomte, 1955). Since these observations were made it has also been shown that the histamine releaser 48/80 releases catechol amines from isolated chromaffin granules (Högberg and Hillarp, 1956).

In the series of aliphatic monoamines, the relationship between length of the polymethylene chain and potency as a releasing agent was similar to that described by Mongar and Schild (1953) for their activity in releasing histamine from guinea-pig lung.

Another similarity between the ability of these substances to release histamine on the one hand, and catechol amines on the other, was in the effect of *p*H. An increase in the histamine-releasing activity of these aliphatic amines, with both increasing and decreasing *p*H, was reported to the British Pharmacological Society in January, 1956, by Mongar, who interpreted his observations by assuming that the histamine-carrying granules were surrounded by a membrane, which was permeable to the releasing amine in its unionized form, whereas the releasing effect was exerted within the granule by the ionized amine. A similar mechanism must also be postulated for the chromaffin granules, and it is of interest in this connexion that electron-microscopic investigation of the chromaffin granules has shown that they are in fact surrounded by a membrane (Lever, 1955; Sjöstrand and Wetzstein, 1956).

Whereas the mechanism of release of both histamine and catechol amines from isolated granules appears to be similar, it is interesting that in experiments on intact animals the substances tested are mainly active as releasers of histamine. This could be explained by assuming that there exists a difference in the cell membranes of amine-carrying cells. The cells which store histamine, such as the mast cells, may be characterized by a particularly vulnerable cell membrane, which allows the releasing agents to act upon the granules in the intact cell, whereas the granules in the chromaffin cell are less readily accessible. This would explain why the action of these substances upon the chromaffin granules becomes manifest only after the granules have been isolated.

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