Biochemical Pharmacology, Vol. 16, pp. 2095–2100. Pergamon Press Ltd. 1967. Printed in Great Britain

# THE FATE OF THE CHROMAFFIN GRANULE DURING CATECHOLAMINE RELEASE FROM THE ADRENAL MEDULLA—I.

## UNCHANGED EFFLUX OF PHOSPHOLIPID AND CHOLESTEROL

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(*Received* 14 *March* 1967; *accepted* 7 *May* 1967)

**Abstract**—Bovine adrenal glands were perfused *in vitro* or *in situ* with Locke's solution and were stimulated with acetylcholine (ACh). Catecholamine efflux rose sharply in response to acetylcholine but there was no change in the efflux of phospholipids or cholesterol, substances that are known to be present in the membranes of the chromaffin granules. The results support the view that the membranes of the chromaffin granules remain in the cell during catecholamine extrusion.

ALTHOUGH the mechanism of secretion (in the sense of extrusion or release) of catecholamines from the adrenal medulla has been the subject of experimental enquiry for many years, the cellular events involved remain uncertain. In the older work, based entirely on light microscopy, the suggested mechanisms have encompassed holocrine and apocrine secretion as well as more subtle forms of release not involving disruption of the cell.[1] When, some years ago, cell fractionation revealed what appeared to be a sizable pool of catecholamines "free" in the cytoplasm of chromaffin cells, the suggestion was made that during stimulation this free amine might escape.[2–4] However, from these same cell fractionation experiments and from electron microscopy it is clear that the bulk of the catecholamines within the chromaffin cells is stored in the now familiar membrane-limited chromaffin granules, and another set of hypotheses holds that it is these granules that are involved in the secretory response. Support for this view has recently been provided by experiments showing that when the chromaffin cells are stimulated to release catecholamines, other substances known to be present in the chromaffin granules are also discharged. The first evidence of this sort was provided by the discovery of ATP and its metabolites in the venous effluent from cats' adrenal glands stimulated through their nerves or with ACh. In these experiments the molar ratio of catecholamines to ATP and its metabolites was similar to that found in the chromaffin granules.[5–7] Then it was found that protein immunologically identical with soluble protein present in chromaffin granules also escapes on medullary stimulation.[8,9] If the chromaffin granules are indeed the source of the catecholamines, nucleotides, and protein released from the secreting chromaffin cell, the question that now arises is: How does release occur? Are the granules extruded intact into the vasculature as Cramer[10] and others[1] have proposed? Or are the various constituents released pre-

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ferentially leaving behind the granule membrane as some electron microscopical evidence suggests.[11–13]

If whole chromaffin granules are extruded, then phospholipid and cholesterol, the major constituents of the membranes of these granules, should appear in the effluent from secreting adrenal medullae. The present experiments were designed to examine this possibility.

## METHODS

*Perfusion of adrenal glands* in vitro. Bovine adrenal glands obtained from the local slaughterhouse and weighing 8–12 g were placed on ice within a few minutes of their removal from the animal, and were prepared for perfusion within 2 hr by a modification of the method of Hechter *et al.*[14] A polyethylene cannula was placed in the adrenal veins and the glands were perfused in retrograde fashion at room temperature (22–25°) with Locke's solution at a pressure of about 100 mm Hg. Multiple incisions were made on the surface of the gland with a razor blade without penetrating to the medullary tissue, and this resulted in a flow of about 20 ml/min of perfusate. Perfusion was continued for 40 min before any samples were collected in order to allow catecholamine efflux to reach low and steady values and to wash out the blood. Catecholamine secretion was evoked by switching perfusion from Locke's solution to Locke's solution containing ACh, $10^{-5}$–$10^{-4}$ g/ml, for periods of 1–3 min. When glands were stimulated more than once, at least 10 min was allowed between stimulations. The composition of the Locke's solution used was as follows (mM): NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; Mg $Cl_2$, 1·0; $NaHCO_3$, 6·0; and glucose, 10·0. This solution was equilibrated with 5% $CO_2$ in $O_2$.

*Perfusion of adrenal gland* in situ. A 4-week-old calf weighing 60 kg was anesthetized with sodium pentobarbital (30 mg/kg, i.v.). After evisceration, the left adrenal gland was perfused via the aorta with Locke's solution at room temperature (25°) and at a pressure of 100 mm Hg. The adrenal effluent was collected through a polyethylene cannula inserted into the adrenal vein; the flow through the gland was about 10 ml/min. Catecholamine secretion was evoked with Locke's solution containing ACh, $2 \times 10^{-5}$ g/ml, for 3 min. This stimulus was applied three times at 10-min intervals, and samples of perfusate were collected each minute.

*Cell fractionation.* Chromaffin granules from bovine adrenal glands obtained from the slaughterhouse were prepared by the method of Banks[15] with the following modifications: the 600 *g* supernatant was recentrifuged for 20 min at 600 *g* to eliminate any sediment carried over in decanting; and the 5000 *g* sediment was resuspended in 0·3 M sucrose and recentrifuged at 5000 *g* for 20 min to further reduce the possiblity of microsomal contamination before layering on the density gradient. After centrifugation, the opaque band in the 1·8 M sucrose layer was separated by aspiration and assayed for catecholamines, phospholipids, cholesterol, and ATP. The ATP measurements were made to allow comparison of the granules obtained in the present experiments with those obtained by Banks.[15] In six experiments the molar ratio of catecholamines to ATP was 4·87 $\pm$ 0·42. This agrees closely with Banks' figure of 4·8.

Chemical determinations were made on control samples of perfusates collected in the 1- to 3-min period before each stimulus and on samples collected during exposure to ACh. In a few experiments serial samples were collected during and after stimulation.

Catecholamines (adrenaline plus noradrenaline) in the perfusates and in perchloric

acid extracts of chromaffin granules were assayed by the trihydroxyindole method.[16] ATP in granule extracts was determined by the firefly luminescence technique as previously described.[6,7] Chloroform–methanol extracts[17] of perfusates and chromaffin granules were assayed for phosphorus[18] and cholesterol.[19] The phospholipid content of the samples was calculated by multiplying the phosphorus values by 25. Protein in perfusates was determined spectrophotometrically[20] after dialyzing samples for 48 hr.

## RESULTS

### *Phospholipid and cholesterol in chromaffin granules*

A knowledge of the amount of phospholipid and cholesterol present in the chromaffin granules is required before the rates of efflux of these substances can be used to define the cellular mechanisms involved in catecholamine extrusion.

Hillarp[21] reported that the mean ratio, by weight, of catecholamines to phospholipid in the chromaffin granules of the ox was 1·19. However, with the present method, which gives a more highly purified chromaffin granule fraction, the value obtained was 2·39 $\pm$ 0·16 (28 different preparations).

There is no published value for the ratio of catecholamines to cholesterol in chromaffin granules, but Blaschko *et al.*[22] reported that the molar ratio of cholesterol to phospholipid in bovine chromaffin granules is 0·7. This value taken in conjunction with the ratio, by weight, of catecholamines to phospholipid that we have observed (2·39) yields a calculated ratio, by weight, of catecholamines to cholesterol of 6·6. In the present experiments the observed ratio was 6·76 $\pm$ 0·57 (9 preparations).

### *Efflux of phospholipids and cholesterol from the adrenal gland*

Sixteen bovine glands were perfused *in vitro* and were stimulated with ACh for 2 or 3 min. Three of the glands were stimulated twice at 10-min intervals so that there were 19 tests in all. Catecholamine efflux in these experiments before exposure to acetylcholine ranged from 8 to 20$\mu$g/min. During perfusion with ACh, the rate increased on the average about 10-fold. Efflux of phospholipids was measured in experiments on 12 of these glands. Before ACh, the values ranged from 15–30 $\mu$g/min and during stimulation there was no significant change. Cholesterol efflux was measured in 6 experiments on 3 glands. The resting values ranged from 7–20 $\mu$g/min, and again there was no significant change on exposure to ACh (Table 1). In a single experiment the efflux of both lipids was measured before ACh, during 3-min stimulation with ACh, and during the 3-min stimulation that followed. The efflux of the two lipids remained at about control levels throughout the experiment, although catecholamine output increased more than 30-fold during exposure to ACh (Fig. 1).

To determine whether the lack of effect of ACh on the efflux of phospholipids and cholesterol was due to the retrograde method of perfusion, an experiment was done on a calf's adrenal gland perfused *in situ* through its arteries. In each of two tests on this preparation, ACh had no effect on phospholipid or cholesterol efflux. The contrasting effects of ACh on lipid and catecholamine efflux were especially striking in this experiment since the rate of catecholamine output rose more than 700 times during exposure to ACh (Fig. 2, a and b).

In a third test on the same gland perfused *in situ*, protein efflux was measured and

found to increase on exposure to ACh (Fig. 2, c); a similar result was obtained in one of the glands perfused *in vitro* in confirmation of earlier work.[8,9]

TABLE 1. CHANGES IN CATECHOLAMINE, PHOSPHOLIPID, AND CHOLESTEROL EFFLUX FROM BOVINE ADRENAL GLANDS DURING STIMULATION WITH ACETYLCHOLINE*

| Exp. no. | Δ Catecholamines (μg/min) | Δ Phospholipids (μg/min) | Δ Cholesterol (μg/min) |
|---|---|---|---|
| 1 | + 43·4 | + 12·8 | |
| 2 | + 74·3 | − 3·6 | |
| 3 | + 85·0 | + 19·6 | |
| 4 | + 86·0 | − 4·1 | |
| 5 | + 89·1 | + 11·5 | |
| 6 | + 94·2 | − 4·4 | |
| 7 | + 131 | + 3·1 | |
| 8 | + 146 | + 14·9 | |
| 9 | + 189 | − 3·7 | |
| 10 | + 194 | + 0·1 | |
| 11 | + 233 | − 6·3 | |
| 12 | + 237 | + 7·7 | |
| Mean | + 133·5 | + 4·0 | |
| S.E. | ± 18·9 | ± 2·6 | |
| P | < 0·001 | > 0·10 | |
| 13 | + 80·0 | | + 1·0 |
| 14 | + 102 | | + 0·8 |
| 15 | + 104 | | − 0·5 |
| 16 | + 226 | | − 7·0 |
| 17 | + 234 | | − 1·2 |
| 18 | + 269 | | + 2·0 |
| Mean | + 169·2 | | − 0·8 |
| S.E. | ± 33·7 | | ± 1·3 |
| P | < 0·005 | | > 0·5 |

* In all these experiments bovine adrenal glands were perfused in retrograde fashion with Locke's solution at room temperature and ACh ($10^{-5}$ g/ml) was infused for 1–3 min. The values represent the increments (+) or decrements (−) in efflux during exposure to ACh. Student's *t*-test was used to calculate P values.

## DISCUSSION

Up to the present, each of the major constituents of the chromaffin granules that has been sought in the venous effluent from adrenal glands during medullary stimulation has been recovered. Moreover, the amounts recovered suggest that the various constituents are released in the same relative proportions that exist witin the chromaffin granules. This is true of the adenine nucleotides,[5–7] and seems also to be true of the major soluble protein of the granules,[8, 9] although it must be conceded that the immunological assays of protein used do not allow such a precise study of the stoichiometry involved. Clearly then, the present findings are in contrast with the earlier ones, for there was no increase in the rate of efflux of phospholipids or cholesterol during catecholamine release, although chromaffin granules are rich in these lipids. This new evidence appears to rule out those hypotheses of catecholamine secretion which suppose that there is massive extrusion of whole chromaffin granules[10] into the vasculature or that holocrine or apocrine secretion[1] contribute significantly to the acute secretory response. The evidence indicates that chromaffin granules somehow

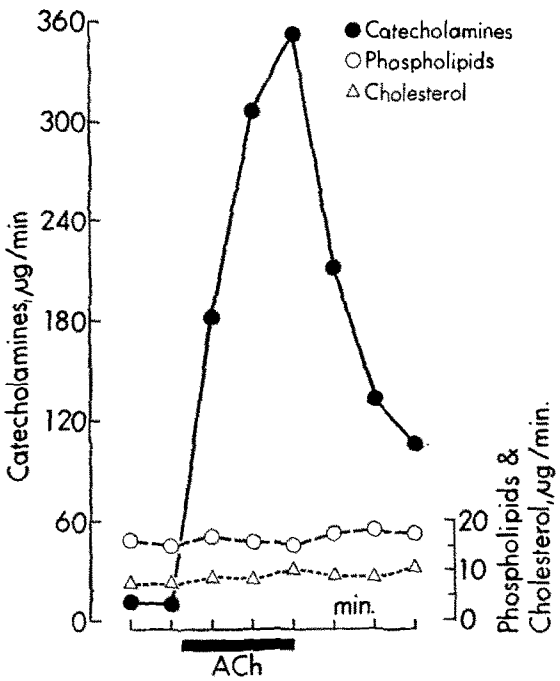


FIG. 1. Efflux of catecholamines, phospholipids, and cholesterol from a bovine adrenal gland perfused *in vitro* through its vein. ACh ($10^{-4}$ g/ml) was given during the period indicated by the horizontal bar.

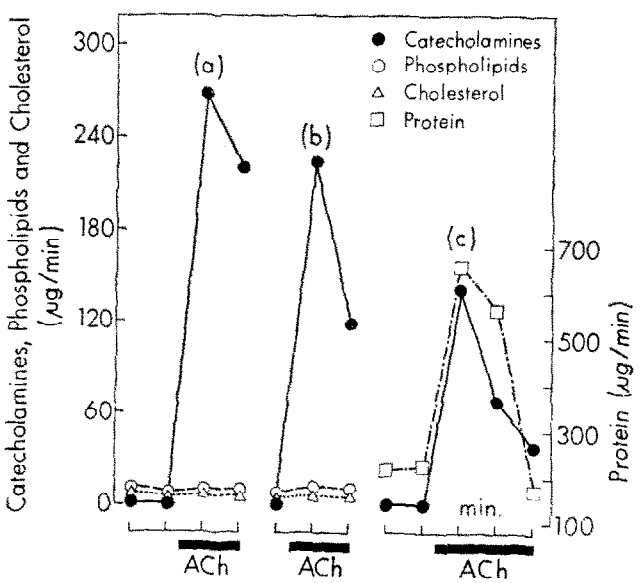


FIG. 2. Efflux of catecholamines, phospholipids, cholesterol, and protein from a calf's adrenal gland perfused *in situ* through its arteries. ACh ($2 \times 10^{-5}$ g/ml) was given during the periods indicated by the horizontal bars. The three responses were obtained at 10-min intervals.

evacuate their content of catecholamines, nucleotides, and proteins without leaving the cell. This interpretation harmonizes with electron micrographs showing what appear to be emptied granules in chromaffin cells that have been stimulated,[11–13] and is consistent with chemical and ultrastructural analyses of subcellular fractions obtained from stimulated glands.[23, 24]

*Acknowledgement*—We thank Miss A. Palazzolo and Mr. A. Hooper for technical assistance. The work was supported by Grants B-4006, 1-K3-GM-25,304 and 5TI-GM-65-09.

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