MOLECULAR PHARMACOLOGY, 10, 678–685

Labeling of Chromaffin Granules in Adrenal Medulla with[^{35}S] Sulfate

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(Received March 6, 1974)

SUMMARY

BAUMGARTNER, HOLGER, GIBB, JAMES W., HÖRTNAGL, HEIDE, SNIDER, STUART R., AND WINKLER, HANS: Labeling of chromaffin granules in adrenal medulla with [^{35}S]-sulfate. *Mol. Pharmacol.* **10,** 678–685 (1974).

The biogenesis of chromaffin granules in adrenal medulla was investigated with [^{35}S]sulfate, which is known to label the sulfated mucopolysaccharides of these organelles. Retrogradely perfused bovine adrenal glands were pulse-labeled with [^{35}S]sulfate. Four hours after the injection of the isotope the adrenal medullae were subjected to subcellular fractionation. It was found that the soluble material which had incorporated [^{35}S]sulfate was specifically confined to cell particles identified as newly formed chromaffin granules. These granules differ from mature ones by their lower density as determined by centrifugation through sucrose gradients. The labeled material appeared to be associated with the acidic chromogranins, as shown by polyacrylamide gel electrophoresis and isoelectric focusing. In experiments with rats *in vivo* it was found that newly formed chromaffin granules which had incorporated the isotope took about 48 hr to mature.

INTRODUCTION

The catecholamine-storing vesicles of the adrenal medulla, the so-called chromaffin granules, are complex organelles. Their content includes both macromolecular components and small molecules, mainly catecholamines, nucleotides, and Ca^{++} (1, 2). The macromolecules are the chromogranins, a group of acidic proteins, and the enzyme dopamine β-hydroxylase (EC 1.14.2.1) (1–3). It seems likely that the intracellular assemblage of such an organelle requires a multistep process.

In an attempt to characterize the events in the biogenesis of chromaffin granules, perfused ox adrenal glands were pulse-labeled with [^{3}H]leucine (4, 5). Four hours after the injection of this amino acid the newly synthesized, and therefore radioactively labeled, chromogranins were found in subcellular organelles which were apparently newly formed chromaffin granules. These particles could be clearly differentiated from mature granules by density gradient centrifugation. The possible conversion of these newly formed chromaffin granules into mature ones could not be established in these studies, since experiments with a gland perfused *in vitro* are necessarily limited in time.

The work presented in this paper was supported by the Fonds zur Förderung der wissenschaftlichen Forschung (Austria) and by the Dr. Legerlotz-Stiftung.

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Experiments on intact animals were therefore warranted.

Recently it has been reported that catecholamine-storing organelles contain sulfated mucopolysaccharides which can be labeled by [^{35}S]sulfate (6–8). We therefore decided to use this isotope for further characterization of the biogenesis of chromaffin granules. The present paper demonstrates that [^{35}S]sulfate labels the soluble content of chromaffin granules of both ox and rat adrenal medulla and establishes that the conversion of newly formed chromaffin granules to mature ones requires about 48 hr.

METHODS

Experiments with Bovine Adrenals

Bovine adrenals were perfused as previously described (4, 5, 9). The perfusion medium was Tyrode's solution plus 10% (v/v) single-strength tissue culture medium 199 (TC 45). Thirty minutes after the start of the perfusion the glands were stimulated once with 15 mM carbamylcholine chloride (9). If a good secretory response was obtained, perfusion was continued, but with the omission of medium 199. Ten minutes later sodium [^{35}S]sulfate (10 mCi) was injected over 3 min into the perfusion medium. Two minutes after the end of the injection medium 199, with the addition of unlabeled sodium sulfate (final concentration, 1 mg/ml of perfusion fluid), was again added to the perfusion medium. After 30 minutes medium 199 without additional sulfate was used. Perfusion was stopped 4 hr after the injection of the isotope. The medullae were dissected out and homogenized with a Potter-Elvehjem homogenizer. The homogenates were subjected to differential and sucrose density gradient centrifugation (120,000 × *g* for 5 hr) as already described in detail (4, 5, 10). The isolated subcellular fractions were diluted with 0.005 M Tris–sodium succinate buffer (pH 5.9), frozen and thawed once, and then dialyzed for 30 hr against the same buffer. After dialysis the fractions were centrifuged at 140,000 × *g* for 40 min in order to separate the soluble and insoluble (membrane) components. Aliquots were either used for enzymatic assay or precipitated with 5% trichloracetic acid. The precipitates were dissolved in *N*-ethyl-*N*-dodecyl-*N*,*N*-dimethylammonium hydroxide in methanol and counted in 14 ml of Bray's solution (11) in a liquid scintillation spectrometer.

For experiments with *N*-cetylpyridinium chloride, dialyzed and undialyzed fractions from the gradient were diluted with 0.01 M Tris-HCl buffer (pH 7.4) to 2 ml. The protein concentration was 150 μg/ml. Varying amounts (15–120 μl) of an *N*-cetylpyridinium chloride solution (20 mg/ml) were then added, which led to visible precipitation of material. After 30 min in ice the mixture was centrifuged for 30 min at 100,000 × *g*. The radioactivity was determined in the supernatant and sediment fractions.

Experiments with Rats

Rats (150–300 g) were given an intraperitoneal injection of 15 mCi/rat of sodium [^{35}S]sulfate. At various times thereafter the rats were killed with chloroform vapor, the adrenal glands were excised, and the fibrous capsules surrounding the glands were removed. The glands of each injected rat were homogenized together with two glands of an untreated rat in 3 ml of 0.3 M sucrose. Nuclei and cell debris were removed by low-speed centrifugation (800 × *g* for 20 min). A large granule fraction was sedimented at 12,000 × *g* for 20 min. The large granules were resuspended in 0.5 ml of 0.3 M sucrose solution. An aliquot (0.45 ml) was layered on top of a gradient ranging from 1.3 to 2 M sucrose solution (10). After centrifugation at 120,000 × *g* for 5 hr, fractions were collected by piercing the bottom of the tube with a needle. Aliquots of the fractions were used for the catecholamine assay. The remainder was diluted with 0.005 M Tris–sodium succinate buffer (pH 5.9). In order to have sufficient protein, 0.2 ml of a soluble lysate of bovine chromaffin granules (4 mg of protein per milliliter) was added to each fraction. After dialysis against the same buffer the fractions were centrifuged for 1 hr at 100,000 × *g* to sediment the insoluble material (membranes). The supernatant fractions were treated with 5% trichloracetic acid. The precipitated proteins were dissolved for liquid scintillation counting as stated above.

Electrophoresis

Polyacrylamide gel electrophoresis (5.6% gels) was performed according to Clarke (12). The electrolyte buffer (pH 8.6) was 0.025 M Tris–0.2 M glycine. For electrophoresis the soluble material from the gradient fractions was concentrated by ultrafiltration through collodion membranes (Sartorius, Germany). Aliquots of the samples were run for 45 min at 80 V and then for 15 min at 160 V. For determining the distribution of radioactive proteins, gels were stained for 10 min in Amido black (1% in 7% acetic acid), then destained for 12 hr in 7% acetic acid. The gels were then cut, according to the localization of the protein bands, into pieces ranging from 0.2 to 0.5 cm. The pieces were treated in scintillation vials with 2 ml of H_2O_2 at 67°, which dissolved the gels overnight. Then 2.0 ml of H_2O and 0.5 ml of *N*-ethyl-*N*-dodecyl-*N*,*N*-dimethylammonium hydroxide in methanol were added. After 2 hr at 67° 14 ml of Bray's solution (11) were added.

Isoelectric Focusing (13, 14)

Gels (4%) were prepared by dissolving 450 mg of Cyanogum in 8 ml of 7 M urea solution. Then 1.125 ml of glycerol, 0.56 ml of Ampholine (pH range, 3.5–10; 40% solution), 0.125 ml of dimethylaminoproprionitrile, and 1.5 ml of ammonium persulfate solution (12 mg) were added. This mixture was poured into glass tubes (Shandon disc electrophoresis apparatus) and then overlayered with water. After polymerization (approximately 2 hr) the gels were subjected to electrophoresis with 1 mamp/gel until the voltage reached 200 V. Electrolyte buffers were 0.01 M phosphoric acid and 0.02 M sodium hydroxide. Then the extensively dialyzed samples were mixed with Ampholine (titrated to pH 8; final concentration in sample, 4%) and applied to the top of the gel. Electrophoresis was started at a constant current of 1 mamp/gel, until the voltage reached 340 V. Then the equipment was switched to a constant voltage of 340 V (usually for about 4 hr). In some experiments electrophoresis was prolonged up to 15 hr, which led to a cathodic shift of the pH gradient but otherwise gave comparable results. After electrophoresis one gel was cut into 0.5-cm pieces, which were eluted with water for measuring the pH. The sample gels were fixed in 12.5% trichloracetic acid. After 30 min the protein bands became clearly visible as precipitates. The gels were then cut to measure the radioactivity as stated above. Before staining the gels were washed repeatedly for 3 days in 12.5% trichloracetic acid, stained for 4 hr in 1% Amido black in 7% acetic acid, and finally destained in the same acid.

Chemical and Enzymatic Assays

Proteins were measured with the biuret reaction as described previously (15). Catecholamines were determined either colorimetrically (16) or by measuring the native fluorescence (17) in perchloric acid (3% final concentrations) extracts of the tissue. Glucose 6-phosphatase (EC 3.1.3.9) was measured according to De Duve *et al.* (18), succinate dehydrogenase (EC 1.3.99.1) according to Porteous and Clark (19), and acid ribonuclease (EC 2.7.7.16) as described by Smith and Winkler (10).

Materials

Sodium [^{35}S]sulfate (50–110 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, England; tissue culture medium 199 (TC 45), from Wellcome Reagents, Ltd., Beckenham, England; *N*-ethyl-*N*-dodecyl-*N*,*N*-dimethylammonium hydroxide, from Merck A.G., Germany; Cyanogum 41, from Serva, Germany; and Ampholine (pH 3.5–10), from LKB, Sweden.

RESULTS

Experiments on Bovine Adrenal Glands

Subcellular fractionation after [^{35}S]sulfate labeling. Perfused adrenal glands were pulse-labeled with sodium [^{35}S]sulfate, and 4 hr later a large granule fraction was isolated and subjected to density gradient centrifugation. Figure 1 gives the results of a representative experiment. Essentially identical results were obtained in two additional experiments. The distribution of marker components in the gradient fractions displays the well-known behavior of lysosomes (acid ribonuclease), of elements of the endoplasmic

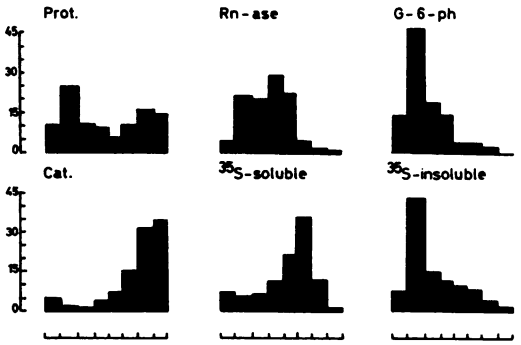

FIG. 1. *Density gradient centrifugation of a large granule fraction from bovine adrenal medulla isolated after pulse of sodium* [^{35}S]*sulfate*

In each histogram the columns from left to right correspond to the fractions from the top to the bottom of the centrifuge tube (sucrose gradient ranging from 1.3 to 2.0 M sucrose). The width of the columns corresponds to the relative volume of the density gradient fractions (total volume of the fractions from one gradient was 4.75 ml). The ordinates give the percentage of the total activity recovered in each fraction, which was obtained by dividing the amount of component per milliliter of fraction by the total amount present in the gradient. The radioactivity in the ^{35}S-labeled soluble material was measured in dialyzed fractions after removal of insoluble material by high-speed centrifugation. The total amount of ^{35}S labeling in the soluble and insoluble material was about equal (27,500 dpm/gradient). The specific radioactivity in the peak fractions amounted to 354 dpm/mg of protein for the insoluble material and 857 dpm/mg for the soluble material. Recoveries from the gradient ranged from 78% to 106%. Prot., protein; Rn-ase, acid ribonuclease; G-6-ph, glucose 6-phosphatase; Cat., catecholamines.

reticulum (glucose 6-phosphatase), and of chromaffin granules (catecholamines). A mitochondrial marker, succinate dehydrogenase (not shown), was distributed in the gradient similarly to glucose 6-phosphatase (cf. ref. 10).

Figure 1 also gives the distribution of soluble and insoluble material labeled with ^{35}S. The soluble material is mainly derived from the content of cell organelles, whereas the insoluble material is representative of membranes. The insoluble components which had incorporated this isotope remained in the top fractions of the gradient. This distribution would indicate localization of the label either in mitochondria or in elements of the endoplasmic reticulum. It seems likely, however, that the label was present in elements of the endoplasmic reticulum, since the microsomal fraction, which consists mainly of these elements, contained 44% of the labeled insoluble material (100% = total amount present in microsomal and large granule fraction). The corresponding figure for glucose 6-phosphatase was 58%.

The soluble material which had become labeled by ^{35}S exhibited a distribution which is apparently different from those shown by the marker components. The labeled material was specifically confined to particles which equilibrated in a position slightly above that taken by chromaffin granules.

Characterization of soluble components labeled by [^{35}S]*sulfate.* The cationic detergent *N*-cetylpyridinium chloride is known to precipitate acidic components like mucopolysaccharides containing sulfate groups (20). Therefore this detergent was used to determine whether the soluble material labeled with [^{35}S]sulfate could be precipitated. More than 90% of the radioactive material present in the dialyzed soluble fractions of the gradient was consistently precipitable.

A characterization of the labeled soluble material by polyacrylamide gel electrophoresis is shown in Fig. 2a. The stained protein pattern is typical of the soluble proteins of chromaffin granules with dopamine β-hydroxylase and chromogranin A, the main component of the acidic chromogranins (cf. refs. 3 and 21). The radioactivity was consistently found spread throughout the gel. There was an increase in radioactive labeling beginning with chromogranin A. The highest level of labeling was present in regions of the gel where the fastest moving components were found. In this region only very faintly staining bands could be seen, which indicates a high specific radioactivity of these fast moving components.

Results of isoelectric focusing experiments are shown in Fig. 2b. The stained protein pattern shows a few minor bands in the less acidic region. The main group of bands is found between pH 4.7 and 5.5. These proteins were identified as the acidic chromogranins.[3] The distribution of radioactivity

[3] H. Baumgartner, J. W. Gibb, and H. Winkler, unpublished observations.

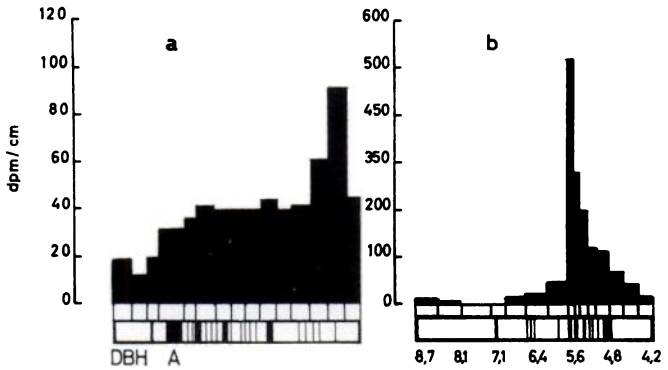

FIG. 2. *Characterization of ^{35}S-labeled soluble material from bovine adrenal medulla*

a. Polyacrylamide gel electrophoresis. The soluble fractions from the density gradient where the peak of labeled material was present (see Fig. 1) were used for electrophoresis. After electrophoresis the gels were stained with Amido black. The stained protein pattern is drawn schematically at bottom. The most slowly migrating band is the enzyme dopamine β-hydroxylase (DBH); the major band represents chromogranin A (A). Afterward the gels were cut into pieces and the radioactivity present was determined. The distribution of radioactively labeled material is given as a histogram drawn above the gel. Recovery of radioactivity from the gel was 101%.

b. Isoelectric focusing. The stained protein pattern and the distribution of radioactively labeled material are given. The pH measured in the various gel regions is indicated. Recovery was 114%.

exhibits a definite peak which coincides with this group of proteins. However, there seems to be a preferential labeling of the less acidic proteins.

Experiments with rats

At various times after the injection of [^{35}S]sulfate the adrenal glands were excised. It proved impossible to remove the cortical tissue completely without loss of medullary tissue. Therefore only the fibrous capsule, with some adhering cortical tissue, was dissected away. Figure 3 gives the results obtained by density gradient centrifugation of large granule fractions in four different experiments. At all time intervals the insoluble material which had incorporated [^{35}S]sulfate remained in the top fractions of the gradient. The soluble [^{35}S]sulfate-labeled material showed a bimodal distribution in the gradient. At all time intervals there was a peak in the top fractions of the gradient, whereas a second peak was found in a position which varied with time. At 5 hr this peak was found between the less dense fractions of the gradient and those containing the bulk of the catecholamines. After 24 hr this labeled material had moved to denser fractions of the gradient. By 48 hr and 96 hr the ^{35}S label was present in a particle which equilibrated in a position very similar to that of the chromaffin granules containing the catecholamines. In contrast to the results obtained in ox glands, the distribution of ^{35}S-labeled material seemed less specific, since considerable material was also found in the top fraction (cf. Fig 1). It is likely that this material is derived from cortical tissue, since it was found that this tissue, when dissected separately, contained a considerable amount of soluble material labeled with [^{35}S]sulfate.

DISCUSSION

Several studies have established that the chromaffin granules from the adrenal medulla contain small amounts of mucopolysaccharides (6, 22, 23). Studies with [^{35}S]sulfate indicated (6, 8) that these components can be labeled *in vivo* with this isotope. In a preliminary communication Fillion *et al.* (6) reported for adrenal medullae of the dog and cat that catecholamines and material labeled after [^{35}S]sulfate injection showed a remarkably similar distribution after centrifugation of homogenates through a density gradient. This apparently indicated specific labeling of catecholamine-storing vesicles. The present study provides detailed information on the subcellular dis-

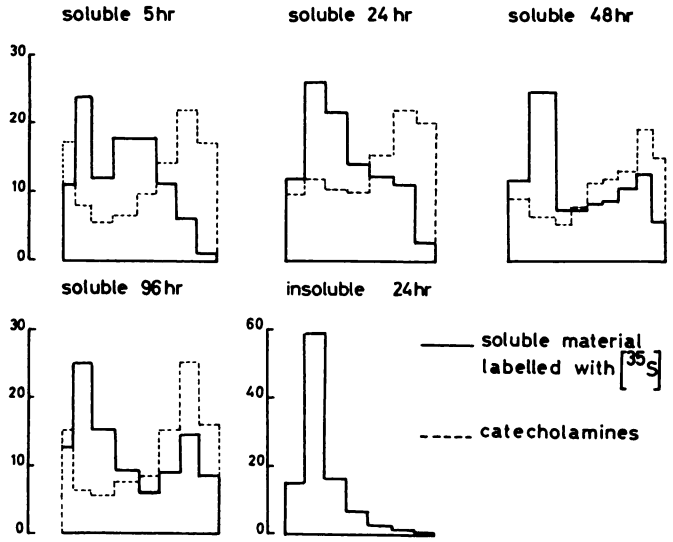

FIG. 3. *Density gradient centrifugation of large granule fractions from rat adrenals*

At varying time intervals after the injection of [^{35}S]sulfate into rats, large granule fractions from the adrenals were subjected to density gradient centrifugation (1.3–2.0 M sucrose solution). The gradient distribution of catecholamines (dashed line) and of ^{35}S-labeled soluble material (solid line) is given for each time interval (for presentation of results, compare with Fig. 1). For the insoluble ^{35}S-labeled material only one distribution is given, since essentially identical results were obtained for all time intervals. The total amount of radioactivity in the soluble material ranged from 438 to 2140 dpm for the various gradients. The corresponding values for the insoluble material are 1191–7700 dpm.

tribution of material labeled by the injection of [^{35}S]sulfate. In perfused ox adrenal glands the ^{35}S-labeled insoluble material was present in membranes of the endoplasmic reticulum, but not in membranes of chromaffin granules. On the other hand, the labeled soluble material was specifically confined to particles which could be differentiated from all other cell organelles by their behavior in a density gradient. They equilibrated slightly above chromaffin granules. In a previous publication (5) we reported that newly formed chromaffin granules which contained newly synthesized chromogranin labeled with [^{3}H]leucine were found in the same position. This would indicate that the ^{35}S-labeled soluble material is present in newly formed chromaffin granules. This suggestion was confirmed by the experiments with rat adrenals *in vivo*. Five hours after the injection of the isotope some labeled soluble material was present in a particle found in a position similar to that of the newly formed granules in the ox experiments. Between 48 and 96 hr later these particles apparently had become mature chromaffin granules. Thus we can conclude that [^{35}S]sulfate became incorporated into the soluble content of chromaffin granules. It should be noted, however, that this isotope is also incorporated into membranes unrelated to chromaffin granules, and in rat glands the soluble components of the cortical tissue also exhibit significant labeling. [^{35}S]Sulfate therefore cannot be considered as specific a marker as the studies of Fillion *et al.* (6) seemed to indicate, although with proper care it can serve as a useful tool.

What is the nature of the soluble components of chromaffin granules labeled by [^{35}S]sulfate? Margolis *et al.* (8) have shown that after labeling of adrenal glands with [^{35}S]sulfate radioactive material can be released by stimulation of the gland with acetylcholine. This agrees very well with our results demonstrating that the soluble content of chromaffin granules was labeled with this isotope. In addition, those authors (8) established that nearly all the labeled material was sulfated mucopolysaccharides. We therefore attempted to define the protein component to which such mucopolysac-

charides are likely to be bound (see ref. 24). We found that the cationic detergent *N*-cetylpyridinium chloride precipitated the labeled material. In addition it has already been shown that this detergent gives quantitative precipitation (21) of the acidic chromogranins, the major soluble proteins of chromaffin granules. Since, however, *N*-cetylpyridinium chloride precipitates both mucopolysaccharides and acidic proteins (20), our results are only consistent with a linkage between chromogranin and mucopolysaccharides but can offer no proof. In electrophoretic experiments with polyacrylamide gels the labeled material seemed to migrate together with the chromogranins. However, the highest radioactivity was present in faster moving components. Two explanations for this behavior are possible. First, the [^{35}S]sulfate-labeled mucopolysaccharides are linked to the chromogranins, and the minor components migrating faster than chromogranin A are preferentially labeled. Second, the ^{35}S-labeled material only migrates with the chromogranins, but is not actually bound to them. Experiments with isoelectric focusing, which separates proteins according to their isoelectric points, favor the first suggestion. The radioactive labeling was confined to a group of proteins focused between pH 4.7 and 5.5. Again minor components seemed to be preferentially labeled. Unpublished experiments[3] with purified chromogranin components have established that these proteins are in fact chromogranins. Thus the results obtained with *N*-cetylpyridinium chloride, polyacrylamide gel electrophoresis, and isoelectric focusing are consistent with the possibility that the ^{35}S-labeled material is linked to the acidic chromogranins. However, since minor components appear to be preferentially labeled, further studies, e.g., biochemical analysis of purified chromogranins, are needed to establish the exact relationship between these proteins and mucopolysaccharides.

The present and a previous study (5) have shown that newly formed chromaffin granules containing chromogranins labeled with either [^{3}H]leucine or [^{35}S]sulfate are less dense than mature ones. For ox adrenal glands perfused *in vitro* the possibility that the low density of new chromaffin granules was an artifact could not be entirely excluded. It was reassuring, therefore, to obtain the same results in experiments under conditions *in vivo*. Furthermore, in the rat experiments longer time intervals after the injection of the isotope could be studied. This enabled us to demonstrate that the conversion of newly formed granules into mature ones takes about 48 hr. Previous studies on rabbits and rats (25, 26) also indicated that newly formed chromaffin granules exhibit a lower density than mature ones. In these experiments the catecholamine stores of the adrenal medulla were severely depleted with insulin, making it possible to detect the population of new vesicles, which otherwise cannot be differentiated from the great number of mature ones. Our studies now show that even in unstimulated glands newly formed vesicles take about 48 hr to mature. It seems likely (see also refs. 25, 26) that these new vesicles are less dense, since they have not acquired their full complement of nucleotides and catecholamines. In this connection it is interesting to note that these vesicles are apparently able to secrete their content. As early as 30 min after [^{3}H]leucine administration the release of radioactively labeled chromogranins could be elicited by stimulation of the adrenal medulla (5). Furthermore, Margolis *et al.* (8) have shown that 24 hr after [^{35}S]sulfate injection labeled mucopolysaccharides could be secreted from the adrenal gland. Thus the adrenal medulla apparently can secrete from granules which do not yet contain their full complement of catecholamines. This seems a rather uneconomical procedure for a gland, the main function of which is considered to be the secretion of catecholamines. Is this secretion from immature granules an expression of the possibility that the chromogranins, like the catecholamines, are secretory products with a hormonal action?

The present study provides further data concerning the biogenesis and maturation of chromaffin granules by using [^{35}S]sulfate as a tool. This isotope is incorporated into the soluble components of chromaffin granules. Thus [^{35}S]sulfate may prove useful for studying the synthesis and transport of catecholamine-storing vesicles even in more compli-

cated structures such as sympathetic nerves or brain tissue. However, the relative lack of specificity of this label must be considered.

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