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SHORT COMMUNICATIONS

Isolation of chondroitin sulfate and glycopeptides from chromaffin granules of adrenal medulla

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RECENT REPORTS have described the chemical composition and metabolism of various classes of mucopolysaccharides and glycoproteins in brain.[1–7] Although very little information is available concerning the functional role of these complex carbohydrates in nervous tissue, it has been suggested that they may be involved in synaptic transmission of the nerve impulse. Consistent with such a role is the report that the concentration of mucopolysaccharides was twice as high in the synaptic vesicle fraction of mouse brain as that found in the whole brain particulate fraction.[8]

It has also been reported that after the administration of labeled norepinephrine or sulfate to dogs, catecholamine and sulfate radioactivity, as well as hexosamine, sediment at the same rate when homogenates from adrenal medulla are centrifuged in a continuous sucrose density gradient.[9] Adrenal medullary cells may be considered as modified postganglionic sympathetic neurons, in which catecholamines are stored in large membrane-bound granules which also contain a high concentration of ATP. It has been concluded that the endogenous catecholamines must be present in the granules in a non-diffusible form, since in experiments with labeled amines no exchange between endogenous and exogenous amines was observed,[10] even though the chromaffin granule membrane is freely permeable to amines and ATP.[11] In view of the possible role of mucopolysaccharides and glycoproteins in the storage and release of neurotransmitter amines at nerve endings, we have examined purified chromaffin granules from adrenal medulla for the presence of these complex carbohydrates.

Chromaffin granules were prepared from bovine adrenal medulla by the method of Smith and Winkler[12] as modified by Foldes *et al.*[13] The chromaffin granules contained an average of 68 per cent of the catecholamines and 7 per cent of the acid deoxyribonuclease present after ultracentrifugation of the large-granule fraction in 1·6 M sucrose, and the molar ratio of catecholamines to ATP was 4·3. These values are very similar to those reported by Smith and Winkler,[12] and for chromaffin granules prepared by sucrose density gradient centrifugation.[14–16]

The pooled granule fractions from 415 adrenals yielded 6·1 g of material after dialysis against distilled water and lyophilization. The lyophilized granules were suspended in 11 ml of water and extracted with 290 ml of chloroform–methanol (2:1, v/v). After drying *in vacuo* over KOH pellets (yield = 3·75 g), 3·7 g of the lipid-free protein residue was suspended in 190 ml of boric acid–borax buffer (pH 7·8) and digested with 100 mg of pronase at 55° as described previously.[3] An additional 100 mg of pronase was added after 24 hr and the digestion was continued for a total of 48 hr, at which time the solution was cooled to 4°, made 10 per cent in trichloroacetic acid, and centrifuged to remove the undigested residue (160 mg, containing less than 2 per cent of the total hexosamine). The protease digest was then neutralized, dialyzed, lyophilized and redissolved in 100 ml of 0.04 M NaCl. Mucopolysaccharides were precipitated with cetylpyridinium chloride and the glycopeptides derived from glycoproteins were recovered from the cetylpyridinium chloride supernatant.[3,17]

Analytical methods for the determination of uronic acid, galactose and fucose have been described previously.[3] Glucosamine and galactosamine were determined using the amino acid analyzer after hydrolysis of mucopolysaccharides for 3 hr in 6 N HCl at 100°, and glycopeptides for 8 hr in 4 N HCl at 100°. Mannose was assayed enzymatically by the method of Finch *et al.*,[18] and sialic acid was determined both by the periodate–resorcinol[19] and thiobarbituric acid[20] methods. Sulfate was measured by the barium chloranilate method of Spencer,[21] acid deoxyribonuclease as described by Smith and Winkler,[12] catecholamines by the method of von Euler and Hamberg,[22] and ATP as described previously.[23]

The mucopolysaccharide fraction isolated by cetylpyridinium chloride precipitation contained 25 per cent hexosamine, of which 96 per cent was galactosamine, and had a molar ratio of hexosamine to uronic acid to sulfate of 1·00:1·04:1·04. The yield of mucopolysaccharide was 54 mg, or 1·5 per cent by weight of the lipid-free protein residue. The material was quantitatively recovered after reprecipitation with cetylpyridinium chloride from 0·3 M NaCl, indicating the absence of unsulfated mucopolysaccharides, and it was digested to the extent of 95 per cent by testicular hyaluronidase.[24]

Electrophoresis on cellulose acetate strips in pyridine–formic acid buffer (pH 3·0) and 0·05 M phosphate buffer (pH 7·0) showed only one spot with the same mobility as authentic chondroitin sulfate. Digestion with chondroitinase ABC and paper chromatography of the resulting unsaturated disaccharides[6] demonstrated the presence of 4- and 6-sulfated disaccharides in a molar ratio of 1·8:1·0. These data allow the identification of the mucopolysaccharides from adrenal medullary chromaffin granules as 61 per cent chondroitin 4-sulfate, 35 per cent chondroitin 6-sulfate, and 4 per cent of a glucosamine-containing sulfated mucopolysaccharide.

In addition to the mucopolysaccharides described above, the lipid-free protein residue from chromaffin granules also contained approximately 5 per cent by weight of glycoprotein carbohydrate. The non-dialyzable glycopeptides obtained by pronase digestion of the chromaffin granule glycoproteins were found to contain a relatively high content of sialic acid (36 per cent of the total carbohydrate) as well as a small amount of sulfate. The carbohydrate composition of these glycopeptides is given in Table 1.

TABLE 1. CARBOHYDRATE COMPOSITION OF GLYCOPEPTIDES*

Sugar	Per cent
Galactose	6·9
Mannose	4·9
Fucose	1·4
N-acetylglucosamine	8·8
N-acetylgalactosamine	3·7
Sialic acid	14·2

* The glycopeptides also contained 0·3 per cent sulfate.

Although the role of the mucopolysaccharides and glycoproteins in chromaffin granules is not known, it is reasonable to suppose that anionic sulfate and carboxyl groups might be involved in the storage and release of biogenic amines in many types of tissues, including at nerve endings. Experimental evidence has been presented in support of such a storage mechanism for mast cell granules and rat thrombocytes, in which histamine and serotonin are considered to be linked by electrostatic bonds to a mucopolysaccharide–protein complex.[25–27] Assuming that both the sulfate and carboxyl groups in mucopolysaccharides could participate in amine binding, the amount of chondroitin sulfate (54 mg) isolated from our pooled preparations of chromaffin granules would contain approximately 0·2 m-moles of anionic binding sites, calculated on the basis of a disaccharide repeating unit with a period weight of 456. Since there is evidence that 4 molecules of catecholamine are complexed with 1 molecule of ATP,[10,28] the number of available binding sites might be as great as 0·8 m-mole, but this is still only 13 per cent of the approximately 6 m-mole of catecholamines which were present in the isolated chromaffin granules. Therefore, even in the possible presence of contamination of the granules with non-granule mucopolysaccharides, the amount of mucopolysaccharide found is much less than adequate to account for the binding of the high concentrations of catecholamines in chromaffin granules. DaPrada *et al.*[29] have arrived at similar conclusions in a recent report on the storage of biogenic amines in blood platelets and adrenal medulla.

It is also possible that sialic acid residues on the glycoproteins of chromaffin granules may be involved in the storage of catecholamines, since they would furnish a number of anionic binding sites approximately equal to those present in the mucopolysaccharides. Chromogranin A, a purified protein which comprises 38 per cent of the soluble protein and approximately 25 per cent of the total protein of the chromaffin granules, has been reported to contain approximately 3·6 per cent by weight of hexosamine and neutral sugars, as well as a very high content of acidic amino acids.[30] Histochemical studies have also indicated the presence of glycoproteins in chromaffin granules[31,32] and their decrease after stimulation of catecholamine secretion.[32] In this connection we have recently found that acetylcholine causes a 12- to 20-fold increase in the release of labeled chondroitin sulfate and heparan sulfate from the cat adrenal perfused *in situ*, together with the expected large increases in catecholamine secretion.[33]

The amine binding capacity of chromogranin A is very low,[34] and according to our findings the mucopolysaccharide and glycoprotein content of the chromaffin granules could each account for the

binding of less than 15 per cent of the catecholamines. However, since all of these substances contain a high concentration of anionic groups, it is possible that some type of structural interaction between the catecholamines and glycoproteins, mucopolysaccharides, or other proteins or low molecular weight constituents of the granules may be responsible for the high amine binding capacity known to occur *in vivo*.

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