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DEMONSTRATION OF A CLASS OF PROTEINS LOOSELY ASSOCIATED WITH SECRETORY GRANULE MEMBRANES*

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Summary

It is shown, in this study, that rat secretory granule membrane preparations, as prepared by the method of Amsterdam et al. [(1971) J. Cell Biol. 50, 187—200], contain a protein fraction which is removed by washing in isotonic medium. This fraction contains unusually high levels of Pro, Gly and Glx, and appears to label rapidly if the rats are pulsed with [14^c] amino acids prior to removal of the glands. The fraction, which may represent specifically adsorbed secretory protein(s) or peripheral membrane protein, is significant to investigators using this model system to study secretory phenomena.

In merocrine cells, the secretory materials are enclosed in membrane-limited packages, the secretory granules. The secretory granule membranes segregate the exportable material from the other cell constituents during the storage of this material and they also appear to play an important role in the release mechanism via fusion with the apical plasmalemma of the cell, followed by rupture at the site of fusion [2—7]. The origin and fate of these secretory granule membranes remain unknown. Jamieson and Palade [8] have shown that transport and packaging of secretory materials occurs even if protein synthesis is inhibited, but their data did not rule out the possibility that resynthesis of secretory granule membrane protein occurs at the same time as synthesis of the contents. Amsterdam et al. [7] pulse-labelled the protein moiety of secretory granule membranes and found that the specific activity approached that of the proteins within the granules, suggesting de novo synthesis of the membrane protein at

* The material reported in this paper was presented, in part, at the 52nd Annual Meeting of the International Association for Dental Research in Atlanta, Georgia, March 1974 [1].

the time of synthesis of contents. The fact that these membrane proteins showed an unusual amino acid composition, high in proline, glycine, and glutamic acid, similar to a family of secretory proteins of the same gland [9], and that these membranes were prepared in hypotonic medium, led us to suspect that the membranes were contaminated with proteins from the secretory granule contents. Therefore, the experiments of Amsterdam et al. [7] were repeated and the effects of washing the secretory granule membranes sequentially with hypotonic, isotonic and hypertonic buffers were observed.

Adult male Sprague-Dawley rats weighing 200—250 g were used in all the experiments. The rats were housed in a light-controlled room (with a 6 AM to 6 PM light period) and fed Purina Lab Chow and given water ad libitum until the evening before the day of the experiment. For each experiment, 8 to 12 rats of the same body weight ± 10 g were fasted overnight and sacrificed the next morning by first anethetizing with ether and then cutting the heart. Both parotid glands were removed from each rat and all were pooled in 0.3 M sucrose homogenizing medium buffered to pH 7.5 with $NaHCO_3$, weighed, and homogenized with a loose-fitting teflon homogenizer according to the method of Amsterdam et al. [7]. A 250 × *g* supernatant fluid of the homogenate was prepared and subjected to sedimentation through a 30% Renographin (Reno-M-60, meglumine diatrizoate, obtained from E.R. Squibb and Sons, Inc.) solution at 3° C in a Spinco SW 25.1 rotor for 15 min at 7000 rev./min, followed by 45 min at 24000 rev./min as described by Kirshner et al. [10]. The pellets obtained by this centrifugation were washed once with 0.3 M sucrose medium to remove residual Renographin, resuspended in 0.3 M sucrose medium, and dialyzed overnight against 10 mM Tris buffer at pH 8.5 containing 0.05 mM EDTA and 0.2 μg/ml $Ph(NPh)_2$ (*N,N'*-diphenyl-*p*-phenylene-diamine) in order to lyse the granules [7]. After dialysis, the fluid in the bag was removed, and the bag was washed twice with 2 ml of dialysis buffer. The washes were added to the material retained within the bag. Secretory granule membranes were separated from the granule contents by centrifugation of this material at 10000 × *g* for 20 min, resulting in pellets containing the membranes, and supernatant fluid containing the granule contents (see Fig.1).

The pellet material (membrane) first was washed twice with the hypotonic buffer (1 mM Tris buffer at pH 8.5 containing 0.05 mM EDTA and 0.2 μg/ml $Ph(NPh)_2$ as described by Amsterdam et al. [7], and the washes were pooled and saved. The membrane fractions were further sequentially washed with isotonic Tris buffer (10 mM Tris pH 8.5, 0.145 M NaCl, 0.05 mM EDTA and 0.2 μg/ml $Ph(NPh)_2$) twice, and then hypertonic buffer (10 mM Tris pH 8.5, 0.5 M NaCl, 0.05 mM EDTA and 0.2 μg/ml $Ph(NPh)_2$) (see Fig.1). Aliquots of the membranes were taken for analyses at each of the following points: after the two hypotonic washes, after the two isotonic washes, and after the two hypertonic washes. The two washes of each type were pooled, concentrated in a Diaflo cell with a UM 10 membrane and also saved for analysis. All the above procedures were done at 0—4° C.

Protein contents of the various membrane fractions were determined by the method of Lowry et al. [11] using crystalline bovine albumin (Armour Pharma-

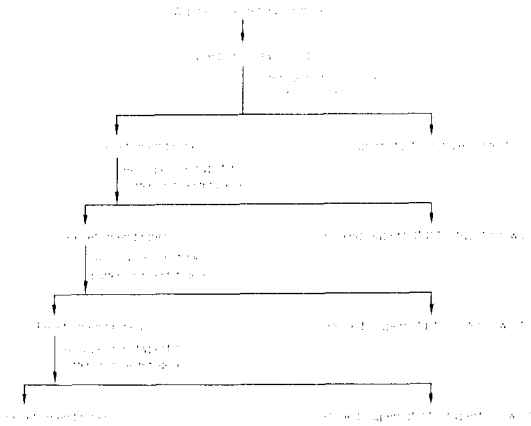

Fig. 1. Scheme for preparing secretory granule contents, the various membrane preparations, and the hypotonic, isotonic and hypertonic membrane washes.

ceutical Co.) as a standard. The succinate dehydrogenase activity and the amylase activity of the 250 × *g* supernatant fluid and the secretory granule fraction were measured according to the methods of Green et al. [12] and Bernfeld [13], respectively. For amino acid analyses of the membrane and wash proteins, aliquots of the samples were subjected to hydrolysis for 18 h in 6 M HCl at 110°C and then analyzed in a Beckman 121 Amino Acid Analyzer.

Sixteen male Sprague-Dawley rats weighing 250 ± 10 gm fed and given water ad libitum until the morning of the experiment. Eight of these rats were anesthetized with pentobarbital sodium (Haver-Lockhart Laboratories) intrapentoneally, and given 12 μC of a mixture of ^{14}C-uniformly labelled L-amino acids (average spec. act. 1.62 mCi/mg, New England Nuclear) in 0.25 ml saline intravenously, using one femoral vein, followed after 15 min with an injection of 6.25 mg of unlabelled amino acid mixture (General Biochemicals of Ohio), into the other femoral vein. The rats were sacrificed 60 min after injection of the labelled amino acids, the parotid glands were removed, homogenized, and secretory granule membranes and contents were prepared as described above. The parotid glands of eight uninjected rats were added to the eight pair of labelled glands in order to provide carrier material during the isolation of the membranes and contents of the granules. The radioactivities of the hypotonically-washed membrane, isotonically-washed membrane, and secretory granule contents were measured by a modification of the method of Madsen [14]. Samples were made 1 M with respect to NaOH, allowed to stand for 1½ h, pipetted into scintillation vials, and neutralized with HCl. 15 ml of a scintillation mixture containing toluene, Triton X-100 and Omnifluor were added and the samples were counted in a Packard Tri-Carb liquid scintillation spectrophotometer. The protein content of aliquots of the same samples were measured by the method of Lowry et al. [11], and the radioactivity expressed as dpm per mg protein after correcting for quenching by use of [^{14}C] toluene internal standard.

The secretory granule preparations were found to contain 25.6 ± 7.4% (mean ± S.D., n = 5) of the total amylase activity, and 3.0 ± 0.9% (n = 3) of the total succinate dehydrogenase activity in the 250 × g supernatant starting material. Both these values compare favorably with the results of Amsterdam et al. [7] and Kirshner et al. [10]. The amounts of protein in the various membrane preparations and washes, relative to the total secretory granule content were as follows: hypotonically-washed membranes, 5%; hypotonic washes, 3%; isotonically-washed membranes, 4.5%; isotonic washes, 0.3—0.5%. The amino acid compositions of the protein moieties of the membranes prepared from these granules, following the various washing procedures, are shown in Table I. The results reported by Amsterdam et al. [7] are shown in the column at the far left. A comparison of these data to those for our hypotonically-washed membranes shows very close agreement. In both cases, Pro, Gly,and Glx levels are high, accounting for approx. 60% of the total amino acid residues.

TABLE I

AMINO ACID COMPOSITION OF SECRETORY GRANULE MEMBRANE PROTEIN

Amino Acid	mol/100 mol			
	Amsterdam et al. [7]	Hypotonically--washed	Isotonically--washed	Hypertonically--washed
Lys	3.2	3.6	5.6	4.1
His	1.3	1.3	1.8	2.0
Arg	5.9	5.6	5.6	5.5
Asx	4.4	5.9	9.2	9.6
Thr	2.1	2.6	4.6	4.8
Ser	2.9	3.6	5.6	6.2
Glx	17.5	18.0	13.9	12.3
Pro	29.8	24.1	11.1	8.2
Gly	17.2	17.0	13.0	13.7
Ala	2.9	3.9	6.5	7.5
½ Cys	—	—	—	—
Val	2.8	2.9	3.7	5.5
Met	0.7	0.6	1.8	1.4
Ile	2.1	2.3	3.7	4.1
Leu	3.7	4.6	7.4	8.2
Tyr	1.4	1.6	2.8	2.7
Phe	1.9	2.3	3.7	4.1
Gly + Glx + Pro	64.5	59.1	38.0	34.2

The amino acid composition of secretory granule membrane is markedly changed by washing the membranes with isotonic buffer, as shown in the third column of Table I. Gly, Glx, and, most remarkably, Pro are reduced, now totalling 38% of all amino acid residues. The proportions of the other amino acids are unchanged or show relative increases. Hypertonic washing of the membranes, subsequent to the isotonic washing, had little, if any, additional effect upon the amino acid composition of the membrane protein.

The amino acid composition of the hypotonic wash solutions (Table II) was found to be very similar to that of content proteins [7]. However, the

TABLE II

AMINO ACID COMPOSITION OF SECRETORY GRANULE MEMBRANE WASHES

Amino Acid	Mol/100 Mol		
	Hypotonic Washes	Isotonic Washes	Salivary Fraction IV
Lys	3.6	1.4	0.9
His	1.9	0.8	3.2
Arg	4.5	5.2	4.5
Asx	9.3	2.2	7.2
Thr	4.5	0.3	0.7
Ser	6.0	1.4	3.6
Glx	13.3	20.4	20.9
Pro	19.5	42.8	32.0
Gly	12.0	21.6	17.9
Ala	4.1	0.7	2.0
½ Cys	—	—	—
Val	5.2	0.7	1.9
Met	—	—	—
Ile	3.4	0.3	0.8
Leu	6.4	0.8	2.2
Tyr	3.2	0.4	1.1
Phe	3.2	0.8	0.9
Gly + Glx + Pro	44.8	84.8	70.8

subsequent isotonic washings were found to contain protein with an extremely high level of Pro (42.8%) and high levels of Gly and Glx, the three amino acids totalling 84.8% of all amino acid residues in this case (see Table II). The right hand column of Table II shows the composition of one family of acidic proteins (referred to as fraction IV) that has been isolated from the saliva and secretory granule contents of this gland [9], and that demonstrates a high Pro, Gly and Glx content.

Since it has already been proposed on the basis of pulse-labelling of rat parotid secretory granule membrane protein, that de novo synthesis of this membrane is concomitant with the synthesis of the secretory materials [7], and since the membranes were prepared in hypotonic medium in that study, these experiments were repeated and the effects of isotonic washing were ob-

TABLE III

SPECIFIC RADIOACTIVITIES OF SECRETORY GRANULE MEMBRANE AND CONTENT PROTEINS

Sample	dpm/mg protein	Results of Amsterdam et al.* [7]
Secretory Granule Contents	2584 (100%)	470 (100%)
Hypotonically-washed membranes	1939 (75%)	390 (83%)
Isotonically-washed membranes	1166 (45%)	

*Expressed as CPM/mg protein

served. As shown in Table III, 60 min following the administration of ^{14}C-labelled amino acids, the hypotonically-washed secretory granule membranes exhibit 75% of the specific activity of the granule contents protein. This compares favorably with the results obtained by Amsterdam et al. [7] with their