Biochemical and Morphological Studies of Rat Submandibular Gland: I. Centrifugal Fractionation of Granule-Rich Fraction

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Submandibular glands from male rats were homogenized in 0.34 M sucrose and 0.5 mM ethylenediaminetetraacetic acid in 10 mM HEPES buffer at a pH of 7.4. The extract was centrifuged and filtered through nuclepore filters to prepare a granule-rich fraction. Electron dense zymogen granules constituted approximately 85% of the particles in this fraction which also contained about a third of the total alkaline esterase activity in the gland.

In addition to protein digestive properties, trypsinlike proteolytic enzymes of rodent submandibular glands (SMGs) have been shown to promote vascular permeability, smooth muscle contraction, increased blood pressure, and growth of a number of embryonic and cultured tissues.[1-5] Most research in this area has dealt with isolation and purification of soluble proteins from whole gland homogenates. A few investigators have isolated the granule-rich fractions from either rat or mouse SMG homogenates and have shown evidence that the granules are responsible for a major part of the peptidase and other biologic activity of this gland.[3,6,7] In addition, the progressive accumulation of alkaline protease activity has been associated with the maturation of rodent SMGs.[8] Presence of this enzyme activity appears to be related to differentiation of the ductal system and to be under the influence of such endocrine factors as testosterone and thyroxin.[8-11]

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It is necessary to isolate and purify individual proteins in order to study the relationships between the alkaline esteropeptidases (AEPS), their biologic activities, and the growth and development hormones. Therefore, this article describes, as a first step, the isolation of organelle-rich fractions from rat SMG homogenate by differential centrifugation and determination of the relative amounts of AEP activity in each subcellular fraction.

Materials and Methods

PREPARATION OF CENTRIFUGAL FRACTIONS.—Adult male rats weighing between 400 and 500 gm each were killed by cervical dislocation and exsanguination through the inferior vena cava. The submandibular glands were immediately removed, dissected free of connective tissue, minced with razor blades, and homogenized in a glass Potter-Elvehjem mortar[a] (30 ml) with a fitted Teflon pestle turning at maximum speed on a stirring apparatus[b] for three up-and-down strokes. This was followed by three more up-and-down strokes of a loose, Dounce homogenizer[a] (41 ml) fitted with a loose glass pestle. The homogenate was then filtered through four layers of clean cheesecloth to remove fibrous connective tissue and large tissue debris.

The fractionation scheme used is shown in Figure 1. All steps were done at 0 to 4 C on an ice bath. The homogenizing medium consisted of 0.34 *M* sucrose and 0.5 m*M* ethylenediaminetetraacetic acid (EDTA) in 10 m*M* HEPES buffer, with a pH of 7.4. The glands were homogenized as a 5% (w/v) suspension, and the homogenate was

[a] Arthur Thomas Co., Philadelphia, Pa.

[b] Model 134-2, Talboy Engineering Co., Emerson, NJ.

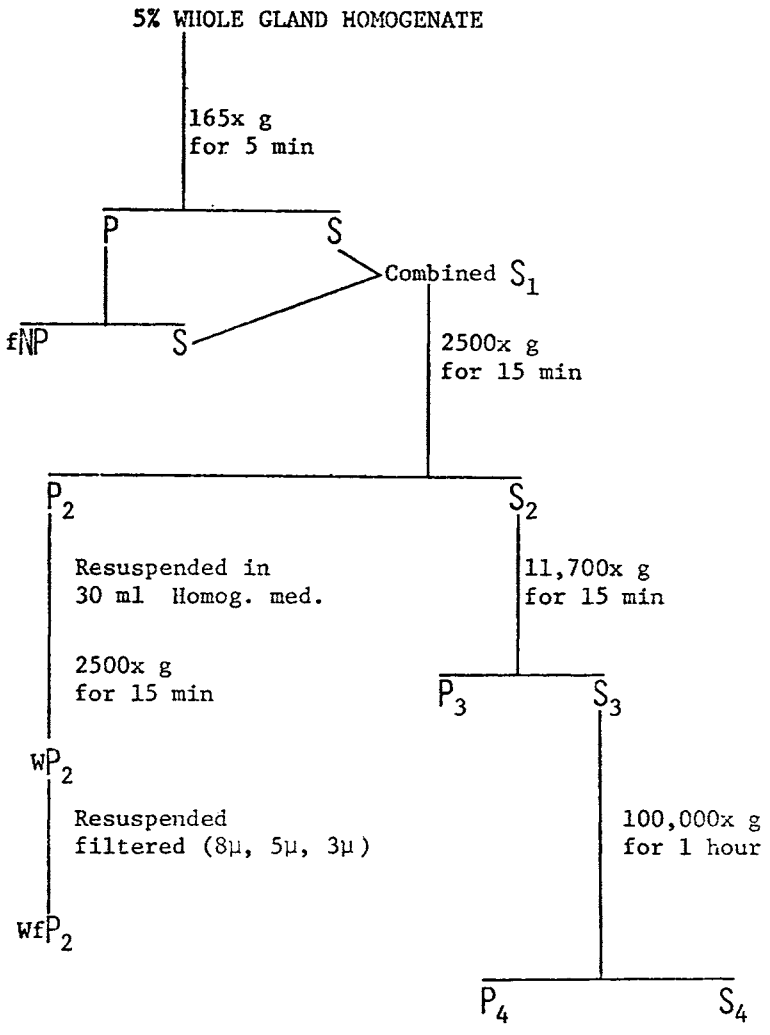

FIG 1.—Centrifugal fractionation and filtration scheme for homogenized rat SMG.

centrifuged[c] for five minutes at 165 × g. The pellet was suspended in a volume of homogenizing medium equal to the supernatant removed and centrifuged again under identical conditions. The combined post-nuclear supernatant (S_1, a 2.5% gland suspension) was centrifuged at 2,500 × g for 15 minutes to prepare a single, crude, granule-rich fraction (P_2). P_2 was then washed with 30 ml of homogenizing medium and filtered through 8-, 5-, and 3- micrometer (μm) nuclepore filters,[d] successively, to obtain a purer granule-rich fraction (wfP_2). Fractions were frozen in liquid nitrogen and stored at −70 C for subsequent biochemical assays.

The supernatant (S_2) resulting from 2,500 × g centrifugation was then centrifuged at 11,700 × g for 15 minutes to separate a mitochondria-rich fraction (P_3) from the post-mitochondrial supernatant (S_3). The S_3 fraction, in turn, was centrifuged at

[c] Sorvall RC2-B centrifuge.

[d] Arthur H. Thomas Co., Philadelphia, Pa.

100,000 × g for one hour in a preparative ultracentrifuge[e] using a high-speed rotor[f] for preparation of a microsome-rich pellet (P_4) and final supernatant (S_4).

BIOCHEMICAL ASSAYS.—Esterase activity was measured using as substrate, 0.5 m*M* BAEE,[g] in 0.1 *M* glycine-NaOH buffer (pH, 9.2) or 0.1 *M* Tris buffer (pH, 6.0). The esterase activity was determined by the rate of change of absorbance (with time) at a 253-nm wavelength with use of a double-beam recording spectrophotometer[h].[12]

For measuring peptidase activity, BAPA[i] was used as a substrate. The incubation mixture consisted of 1 ml of 0.1 *M* glycine-NaOH buffer with a pH of 9.2 (or 0.1 *M* Tris-HCl buffer with a pH of 8.0), 0.5 ml of sample solution, and 0.5 ml of 1 m*M* BAPA (in the same buffer). After incubation at 37 C for the appropriate time (15 to 60 minutes), the reaction was terminated by the addition of (and mixing) 1 ml of 1 *M* sodium acetate-HCl buffer, with a pH of 4.2. The amount of paranitroaniline liberated was determined by measuring the increase in absorbance at 410 nm with use of a spectrophotometer[j] against a reagent blank treated similarly (except that 0.5 ml of buffer was used instead of enzyme solution in the blank cuvette). A calibration curve was prepared using paranitroaniline and the same experimental conditions, according to Ekfors, Malmiharju, and Hopsu-Havu.[13]

Acid phosphatase (EC 3.1.3.2) activity in various fractions was determined at 37 C and at a pH of 6.0 by sodium phenolphthalein hydrolysis according to the method of Huggins and Talalay.[14] Succinoxidase activity was determined by measuring the formazan products resulting from electron transfer from several "mitochondrial" enzymes to nitro blue tetrazolium according to the method of Nachlas, Margulis, and Seligman.[15] Glucose-6-phosphatase (EC 3.1.3.9) activity was assayed by measuring the amount of inorganic phosphorus released from the substrate after incubation with the fractions according to the method of Fiske and Subbarow.[16]

[e] Spinco model L2-65B, Beckman Instruments, Inc., Fullerton, Calif.
[f] SW27, Beckman Instruments, Inc., Fullerton, Calif.
[g] *N*-benzoyl-*L*-arginine ethyl ester-hydrochloride.
[h] Bausch and Lomb Spectronic 505, Bausch and Lomb, Rochester, NY.
[i] *N*-benzoyl-*DL*-arginine-*p*-nitroanilide hydrochloride.
[j] Bausch and Lomb Spectronic 70, Bausch and Lomb, Rochester, NY.

The method of Lowry et al[17] was used to determine protein concentrations. Total enzymatic activity was expressed as the number of micromoles of substrate hydrolyzed per minute per fraction, and specific activity was expressed as the number of micromoles of substrate hydrolyzed per minute per milligram of protein.

LIGHT AND ELECTRON MICROSCOPY.—The content of pelleted material from each step of the fractionation procedure was quickly evaluated by spreading a drop of suspension on a clean glass slide and staining it with 1% toluidine blue in a 1% sodium borate solution. In this manner, nuclei, mitochondria, secretory granules, and microsomes could be distinguished at the light microscopic level. Fresh pellets were also suspended in 2.5% glutaraldehyde in 0.1 *M* sodium phosphate buffer (pH, 7.2) for a period from two hours to overnight at 4 C. After pelleting by centrifugation, the fractions were postfixed in 2% osmium tetroxide for two hours. Rapid dehydration was done either in an acetone series or an ethyl alcohol-propylene oxide series, and was followed by embedding in epoxy resin[k].[18,19] Representative blocks from pellets and segments of pellets were sectioned at approximately 0.5 μm for light microscopy and at approximately 0.1 μm for electron microscopy. Toluidine blue was used for staining the 0.5 μm sections on glass slides, whereas uranyl acetate[20] and lead citrate[21] were used for staining the 0.1-μm sections on formvar-coated or uncoated copper grids (200- and 300-mesh). Thin sections were studied with an electron microscope[l] at 60 kv accelerating voltage.

To determine the approximate number of different organelles in typical centrifugal fractions, electron micrographs were taken at a magnification of × 500. A glass grid 6 × 6.5 cm was then placed over negative images of the fractions and the numbers of each type of organelle were tallied per a 39-cm² area of negative (representing approximately a 150-μm² area of unmagnified pellet). Standard error of the mean was calculated for the P_2 fraction only since the strands and particles of endoplasmic reticulum became so numerous as to be uncountable in fractions P_3 and P_4.

Supernatant fractions were handled simi-

[k] Ladd Research Industries, Inc., Burlington, Vt.
[l] Model 9A, Carl Zeiss, Inc., New York, NY.

larly to pellet fractions except that equal volumes of 5% buffered glutaraldehyde were added to the supernatant for overnight prefixation before centrifugation. Osmium tetroxide postfixation, dehydration, and embedding were done routinely as just described.

Several P_2 fractions were viewed under the scanning electron microscope in an attempt to distinguish between mitochondria and secretory granules and to evaluate the surface characteristics of the secretory granules. These fractions were prepared by spreading the pelleted material on nuclepore filters, fixing them in sodium phosphate buffered 2.5% glutaraldehyde, and quickly dehydrating them in acetone. The dried filters were then placed on a metal specimen block, coated with a 150 A gold film, and scanned at a magnification of × 3,000 under a scanning electron microscope[m] at 15 kv.

HISTOCHEMISTRY.—Slices of SMGs and sublingual gland were fixed in acetone and incubated with the trypsin substrates, BAPA and BANA.[n] The combinations of methods that gave the best results for the two substrates are described. The tissue was fixed in 100% acetone at 4 C for 18 to 24 hours, then subjected to a toluene dehydration series from 30 to 100% at 30-minute intervals on an ice bath, followed by vacuum embedding in paraffin (melting point, 5 C). This method proved superior to the following methods of fixation and dehydration: (1) 4% Formalin in a 0.1 *M* phosphate buffer followed by ethanol dehydration, (2) 6% glutaraldehyde in 0.1 *M* phosphate buffer followed by ethanol dehydration, (3) 95% ethanol followed by xylene dehydration, (4) or freeze-drying in liquid nitrogen after allowing tissue to remain for several hours in 0.88 *M* sucrose, 10% dimethylsulfoxide (DMSO) or 5 to 20% polyvinyl alcohol for subsequent cryostat microtomy.

After embedding, the paraffin sections were cut at a 5 μm thickness, placed on glass slides, and incubated at various temperatures from 4 to 52 C in coplin jars, according to the method of Hopsu and Glenner.[22]

For the substrate BAPA, the incubating mixture included: 1 ml of 1.1 m*M* BAPA and 9 ml of 0.1 *M* buffer (Tris-HCl with a pH of 8.2; glycine-NaOH with a pH of 9.2). A positive reaction was evident within five minutes in the reaction vessel at room temperature.

For the substrate BANA, a postcoupling reaction was most efficient. The hydrolysis incubation mixture included: 1 ml of 1.1 m*M* BANA (solubilized first in 0.1 ml DMSO and then diluted in water) and 9 ml of 0.1 *M* buffer (Tris-HCl, with a pH of 8.2; glycine-NaOH with a pH of 9.2). This incubation reaction was allowed to proceed for 15 minutes at room temperature after which the slides were placed in another coplin jar for coupling. The coupling solution contained 1 ml of dye stock (3.5 fast Garnet GBC/ml distilled water), 1 ml of 10% Tween 20, and 8 ml of 0.1 buffer (same buffer as in hydrolysis reaction). The coupling reaction took place within five minutes at room temperature.

Results

LIGHT MICROSCOPY.—Acetone-fixed sections of normal rat SMG when incubated with BANA and postcoupled with fast Garnet GBC or when incubated with BAPA gave

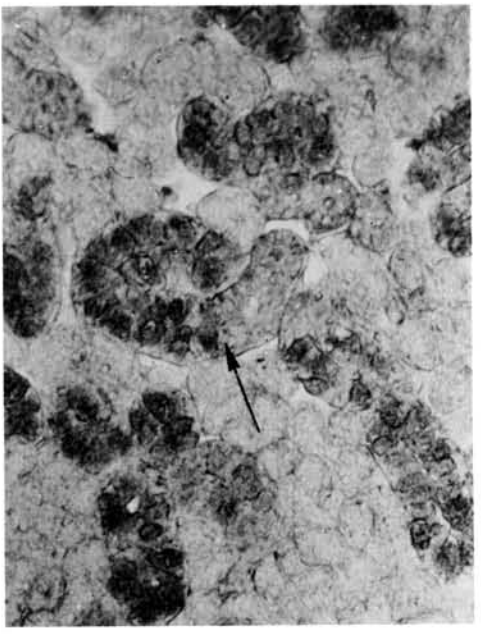

FIG 2.—Photomicrograph of acetone-fixed, paraffin-embedded rat SMG incubated with 0.11 m*M* BANA (pH, 9.2) and postcoupled with fast Garnet GBC. Darker staining structure (arrow) represents convoluted tubules (mag ×200).

[m] Model JEM P3, Jeolco (USA), Inc., Medford, Mass.
[n] N-benpoyl-*DL*-arginine-b-naphthylamide hydrochloride.

identical and characteristic reaction patterns (Fig 2). The dark-brown reaction product was, in all instances, localized over the area of the ductal system of the SMG which is consistent with the pattern of the convoluted granular tubules under routine staining conditions (Fig 3). No reaction product was localized over the ductal system area of the sublingual gland in control sections. When pellets derived from steps in the centrifugal fractionation procedure were stained with toluidine blue, the granules were easily distinguishable from background particulate (mitochondria) and stringy material (microsomes) to which they were adherent. When such a pellet was observed in thick sections cut from epoxy resin blocks[n] prepared for electron microscopy by routine methods, the

[n] Epon 812, Ernest F. Fullam Inc., Schnectady, NY.

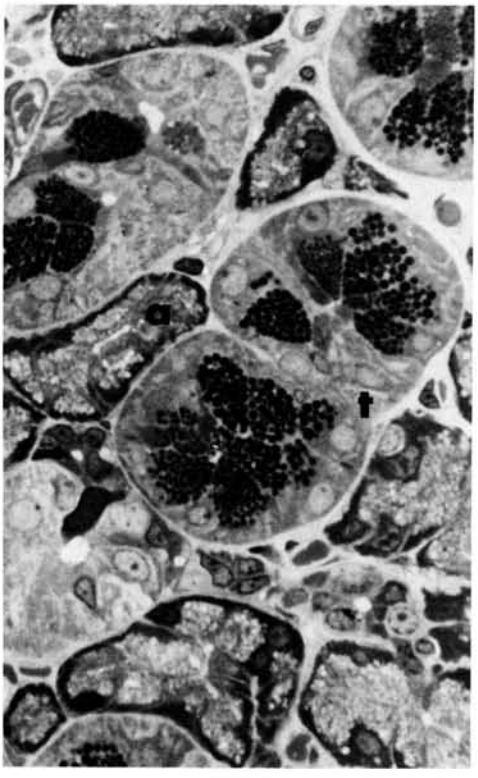

FIG 3.—Photomicrograph of epoxy resin-embedded section of adult male SMG shows convoluted tubules (t) and acinar structures (a). Cells of convoluted tubules vary as to number of dense granules they contain, as well as size and staining qualities of these granules (mag ×650).

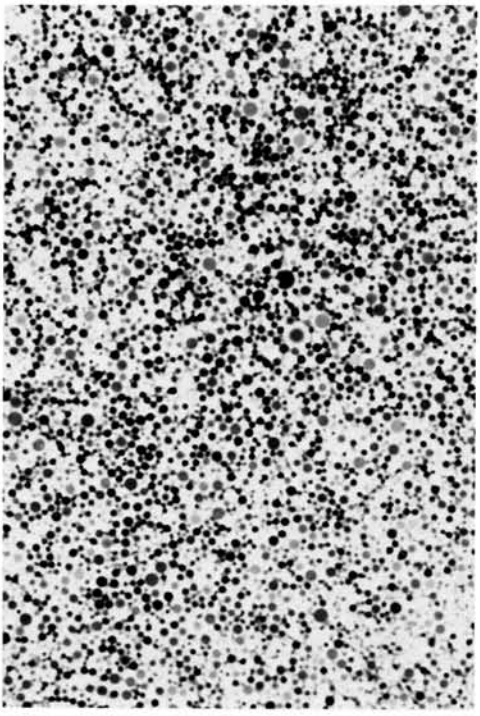

FIG 4.—Thick plastic section of granule-rich pellet after staining with toluidine blue. Although all of these granules are more dense than acinar cell granules, there is great deal of variation in size and staining qualities of these structures (mag ×750).

P_2 preparation showed variation in size and density of the granules (Fig 4). Each successive filtration of the P_2 fraction through nuclepore filters reduced the amount of contaminating organelles as well as the final yield of the wfP_2 fraction.

ELECTRON MICROSCOPY.—Centrifugation yielded a nuclear pellet that consisted of nuclei, cell membranes, aggregates of cell debris, and connective tissue which were not filtered out by the cheesecloth. The P_2 pellet after washing and filtration through the series of nuclepore filters (wfP_2) consisted mainly of secretory granules with a small amount of contaminating material (Fig 5). A similar preparation observed under the scanning electron microscope consisted of numerous rounded projections lying on top of a coated "holey" 0.8-μm nuclepore filter. However, it was impossible to distinguish between small granules and mitochondria or to visualize the granule surface,

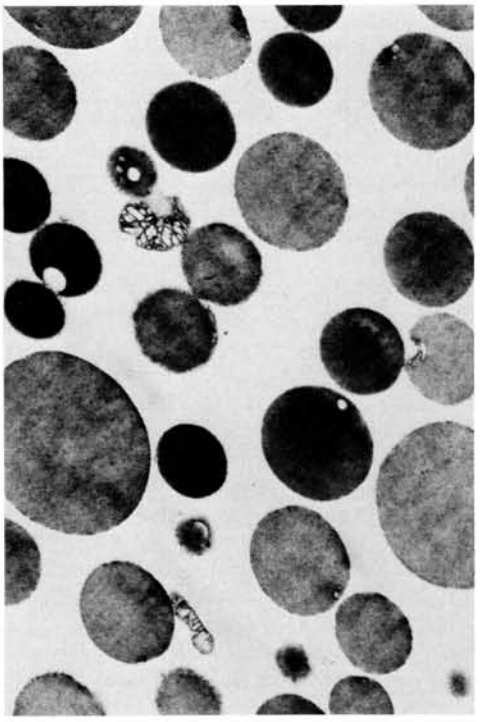

FIG 5.—Electron micrograph of granule-rich pellet shows mainly zymogen-type granules and few mitochondria. Granules exhibit homogeneous matrices, but some are more electron dense than others (mag ×6,500).

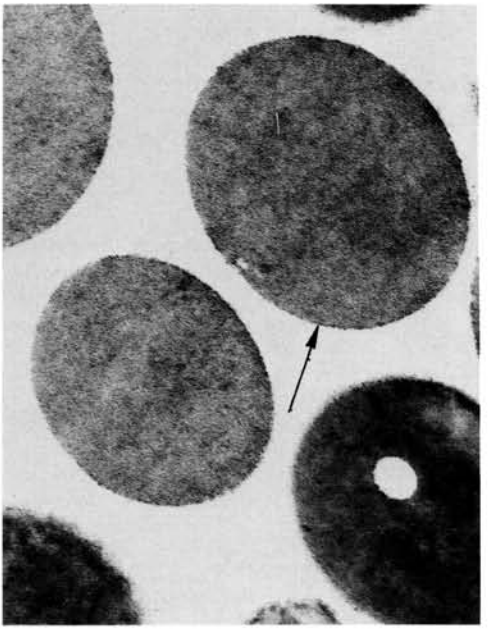

FIG 6.—Zymogen granules with homogeneous matrices are partially surrounded by unit membranes (arrow). No granule was observed in centrifugal fractions after homogenization which were completely surrounded by membranes (mag ×22,500).

especially after coating the preparation with the gold film.

Under the transmitting electron microscope, the wfP_2 pellet consisted of a few mitochondria and many zymogen-type granules, ranging in diameter from 0.8 to 2.5 μm. In different P_2 preparations, there was variation in both the extent to which unit membranes surrounded individual granules as well as the density of their matrices (Fig 6). Perhaps this was due to the amount of membrane lost during homogenization and thus the degree to which soluble components could be extracted by the homogenizing medium. In all instances, however, the matrices

TABLE 1

MICROSCOPIC ANALYSIS OF ORGANELLES IN CENTRIFUGAL FRACTIONS

Fraction	Particles ($\bar{x} \pm SEM$) per 150 μm²			Estimate* of Microsomal Membrane Content	Area Viewed (μm²)
	Dense Granules	Mitochondria	Lysosomes		
wfP_2	24.8 ± 1.83 (84.6%) †	3.7 ± 0.86 (12.6%) †	0.8 ± 0.22 (2.7%) †	—	2,100
P_3	0.5 ± 0.85	103 ± 8.52	1.0 ± 0.85	++	300
P_4	. . .	2.3 ± 1.02	. . .	++++++	450

Note: SEM, standard error of the mean; wfP_2, purer granule-rich fraction; P_3 mitochondria-rich fraction; P_4, microsome-rich pellet.

* —, only occasional microsomal membranes; ++, membranes of endoplasmic reticulum between mitochondria; and ++++++, membranes of endoplasmic reticulum too dense to count.

† Percent figure represents the fraction of total particles in the area view of wfP_2.

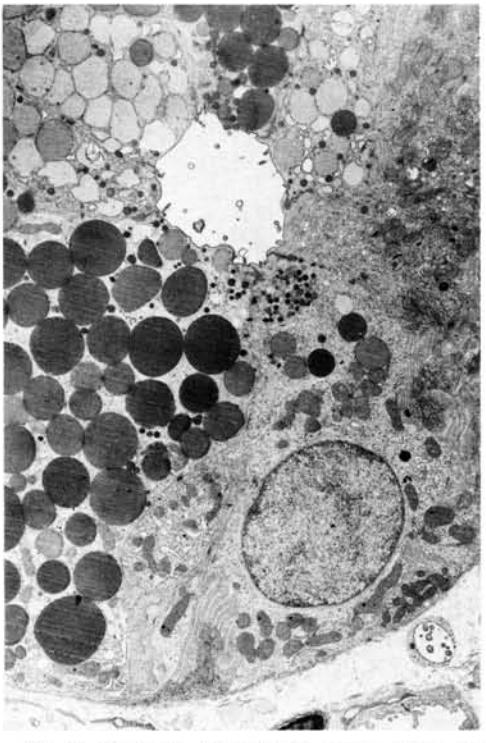

FIG 7.—Electron micrograph shows cross section of convoluted granular tubule. Granules in each cell are similar in density and size, have homogeneous matrices, are surrounded by unit membranes, and are located apical to cell nucleus (mag ×4,000).

of these granules were homogeneous and dense, as in electron micrographs of whole glands (Fig 7), and could be distinguished from adjacent lysomal structures (myelin bodies and autophagic vacuoles). Because acid phosphatase histochemistry was not done on these sections, this impression could not be confirmed. In addition, acinar cell granules with paracrystalline inclusions were rarely present in the preparation. The relative numbers of different kinds of particulate structures in this preparation are shown in Table 1.

The P_3 pellet (centrifuged at 11,700 × g for 15 minutes) consisted mainly of mitochondria but also contained a small number of granules and microsomal structures. The P_4 pellet (centrifuged at 100,000 × g for one hour) consisted mostly of microsomes but was also contaminated with a few mitochondria and "granules."

BIOCHEMISTRY.—Table 2 shows the comparative rates of hydrolysis of BAPA and BAEE at both a pH of 8.0 and 9.2 for centrifugal fractions from a typical experiment according to the preparation scheme. The BAPA hydrolysis rate for each fraction was the same whether the assay was done at a pH of 8.0 or 9.2. The BAEE hydrolysis rates for the various fractions were slightly higher at a pH of 9.2 than rates at a pH of 8.0. In addition, the hydrolysis rate of BAPA was only 0.2 to 0.4% that of BAEE at both pH levels in all fractions. Proteolytic activity in other experiments was followed routinely by the hydrolysis of BAEE at a pH of 9.2.

Table 3 summarizes the esterase activity in the different centrifugal fractions from another experiment. In this instance, more than half (57%) the total activity was found

TABLE 2

COMPARISON OF RATE OF HYDROLYSIS* OF BAPA AND BAEE BY FRACTIONS OF RAT SMG†

	pH, 8.0		pH, 9.2	
	BAPA‡	BAEE§	BAPA	BAEE
Homogenate	104.5	24,570	110.4	32,100
NP (165 × g—15 min)	5.3	2,060	6.4	1,680
P_2 (2,500 × g—15 min)	21.9	8,650	21.9	10,350
P_3 (11,700 × g—15 min)	2.3	875	2.5	1,050
P_4 (100,000 × g—1 hr)	3.7	1,150	3.8	1,150
S_4	56.5	18,200	54.5	18,250
% Recovery	86%	126%	86%	101%

Note: NP, nuclear pellet; P_2, crude, granule-rich fraction; P_3, mitochondria-rich fraction; P_4, microsome-rich pellet; and S_4, final supernatant.

* Total activity (micromoles hydrolyzed per minute per fraction) corrected to original volume of homogenate.

† Adult males (400 to 500 gm each).

‡ α-*N*-benzoyl arginine *p*-nitroanilide.

§ α-*N*-benzoyl arginine ethyl ester.

TABLE 3

DISTRIBUTION OF ALKALINE ESTERASE ACTIVITY IN CENTRIFUGAL FRACTIONS

Fraction	Total BAEE Activity* (μmoles BA Formed/ fraction)	% of Total Activity	Total Protein (mg/fraction)	Specific Activity* (μmoles BA formed/ mg protein)
Homogenate	42,200	100.0	466	90.5
NP	3,660	8.6	91.5	40
P_2	24,100	57	78.1	309
P_3	615	1.5	24.1	25.5
P_4	850	2	38.8	21.9
S_4	14,960	35	204	73.3
% Recovery	. . .	104%	93%	. . .

Note: NP, nuclear pellet; P_2, crude, granule-rich fraction; P_3, mitochondria-rich fraction; P_4, microsome-rich pellet; and S_4, final supernatant.

* The figures represent micromoles α-*N*-benzoyl-*L*-arginine formed per minute at 25 C, with a pH of 9.2.

in P_2. A third of the total activity (32%) remained after washing with homogenizing medium and filtration (wfP_2, not shown in Table 3). This suggests that a large percent of the total esterase activity was associated with organelles in the granular preparation. In addition, it is apparent that BAEE activity with a pH of 9.2 is much less in the P_2 fraction than in the S_4 fraction in Table 2, and it is much more than it is in the S_4 fraction in Table 3. Even with a routine homogenizing and fractionating technique, it was not uncommon for individual preparations to vary to this extent.

Table 4 shows the distribution of marker enzymes (a measure of the degree of contamination) for each fraction. That wfP_2 fraction that had the highest specific esterase activity also had a small percentage of the total acid phosphatase (4%) and total glucose-6-phosphatase (1.4%) activities, indicating little contamination by lysosomes and microsomes, respectively. However, about 20% of the total succinoxidase activity was associated with the wfP_2 fraction thus agreeing with mitochondrial contamination of this fraction.

Discussion

The histochemical activity with BANA and BAPA, when incubated at either a pH of 8.2 or 9.2, was found to be distributed only over the SMG ductal system. The reaction was especially intense over the apex area of these cells, which was reminiscent of the distribution of granules within the convoluted tubules (Figs 2, 3). Other substrates for these enzymes have been used but are less sensitive. For example, the granules of the convoluted tubules react positively in the p-dimethylaminobenzaldehyde reaction[23] for the tryptophane-positive sites.[24,25] In addition, fluorescein-labeled antibodies have been used to localize proteins associated with

TABLE 4

PERCENTAGES OF MARKER ENZYME ACTIVITIES IN CENTRIFUGAL FRACTIONS

Fraction	Percentages*			
	Alkaline Esterase	Acid Phosphatase	Succinoxidase	Glucose-6-phosphatase
Homogenate	100	100	100	100
NP	8.6	20.6	. . .	. . .
P_2	57	6.2	32.3	5.1
wfP_2	32	4.0	19.8	1.4
P_3	1.5	11.1	36.0	21.2
P_4	2	26.0	18.8	88.5
S_4	35	7.8	65.5	<1.0

Note: NP, nuclear pellet; P_2, crude, granule-rich fraction; wfP_2, purer, granule-rich fraction; P_3, mitochondria-rich fraction; P_4, microsome-rich pellet; and S_4, final supernatant.

* Total activity in fractions based on homogenate as 100%.

AEP activity to granules in the convoluted tubules of mouse SMGs.[26]

Since previous studies have strongly suggested that trypsinlike activity, including esteropeptidase activity in the mouse, is related to the granules of the convoluted tubules,[8,11,26] it was expected that most of this enzymatic activity should be associated with a centrifugal fraction rich in zymogen granules. By the simplest fractionation method without any subsequent filtration or gradient manipulation, more than a half the total BAEE activity was found within the P_2 fraction in the experiment illustrated in Table 3. The total activity of this fraction (24,000 μmoles BAEE hydrolyzed/min/fraction) was 57% of the total activity of the homogenate. The final S_4 fraction contained approximately a third of the total activity. A large proportion of this was probably because of breakage of the granules and solubilization of the proteins during the homogenization and isolation procedures. Since 65% of the succinoxidase activity had been found in the final S_4 fraction (Table 4), mitochondria were probably also damaged during this procedure.

It was also apparent that temperature and composition of the homogenizing medium played a major role in the recovery of enzyme activity. In preliminary experiments, 0.88 *M* sucrose, 10 m*M* Tris-HCl buffer, 1 m*M* EDTA, and 1 m*M* lauryl alcohol, at a pH of 7.4 were used as the homogenizing medium, and all steps were done at 25 C. This was a modification of a method described by Geipert and Erdös.[4] Fractionation of the gland after homogenization was easier by this method and the P_2 preparations were less contaminated with other organelles and debris. Therefore, fractionation at room temperature appeared to decrease the tendency of "stickiness" between organelles, perhaps by enhanced proteolysis. However, at the same time, the total activity of the P_2 fraction was reduced to less than 1% of total activity and the specific activity was reduced to less than a quarter of the value reported in this study. More than 90% of the total esterase activity was subsequently and consistently found in the S_4 fraction, suggesting even greater disruption of granules and solubilization of proteins during the homogenization procedures. Disruption of granules and increased vacuolization of granules were confirmed by electron microscopy.

Reikkinen, Ekfors, and Hopsu[27] reported a major soluble enzyme (salivain) with an optimum pH of 9.3 and a specific activity of 1,560 μmoles BAEE/min/mg protein, isolated from whole rat SMG homogenate. In comparison, the highest specific activity reported in the present article was that of the washed and filtered P_2 preparation, 327 μmoles BAEE/min/mg protein, a value which was only about a fifth of that reported by Reikkinen's group. However, their objective was to purify alkaline peptidases from homogenized glands, without regard for their association with organelles. Much of the total activity in the present experiments was lost in attempting to purify a granular fraction.

If the AEP activity is associated with granular proteins of the convoluted tubules as is suggested by these data, then most of the esterase activity at a pH of 9.2 represents salivary enzymes, rather than metabolic proteins associated with other organelles. This parallels the findings of Hopsu-Havu, Reikkinen, and Ekfors[28] who reported that within minutes after intraperitoneal injection of isoproterenol, 0.1 mg/kg, the amidase activity (BAPA) at a pH of 9.2 in saliva was ten times that found in the SMG homogenate. The difference in the sizes and densities of the zymogen granules (Fig 5) may result from differences in composition, that is compartmentalization of different enzymes in separate granules. However, these granules may represent two different stages of maturation, with the enzyme composition being the same in each.

Conclusions

Morphologically, the serous granules of the convoluted tubules of rat submandibular glands were satisfactorily preserved in a homogenizing medium of 0.34 *M* sucrose and 0.5 EDTA in 10 m*M* HEPES buffer at a pH of 7.4 and at 4 C. The preservation as well as yield of granules and total alkaline exteropeptidase activity were better than that with more concentrated sucrose in 10 m*M* Tris buffer at the same pH at room temperature, even in the presence of the membrane-stabilizing agent, lauryl alcohol. Contamination of the granule-rich fraction was mainly by mitochondria. However, it is unlikely that the mitochondria are the source

of these enzymes since such a small proportion of the total AEP activity was found in the mitochondria-rich fraction.

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