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CALCIUM AND SECRETION IN NORMAL AND SUPERSENSITIVE SUBMAXILLARY GLANDS OF THE CAT*

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Abstract—The effect of stimulation of normal and supersensitive cat salivary glands on the subcellular distribution of hexosamine, calcium and protein has been examined. Selective accumulation of calcium and hexosamine in the granular mucin fraction especially of stimulated glands accompanying a greater secretion of hexosamine and calcium indicates that stimulation leads to accelerated hexosamine synthesis with a concomitant increased uptake of calcium into the gland. The possible meaning of the relative amounts of granular hexosamine, calcium and protein are discussed in relation to the formation of hexosamine-rich granules after the secretory stimulus.

CALCIUM has been implicated in the secretion of salivary protein from the submaxillary gland of the cat by Douglas and Poisner.[1] Their evidence points to the permissive role of calcium in the secretory process. Also calcium is taken up by adrenal chromaffin granules as shown by Philippu[2] and Schumann and by Borowitz *et al.*[3] Finally, the denervated submaxillary gland of the cat has been used to demonstrate the supersensitivity of secretion by Emmelin *et al.*[4]

The authors intend to use the above background work to answer the following questions: (1) Is calcium accumulation associated with binding or release of salivary granular mucin? (2) Does the supersensitive salivary gland display any patterns of calcium distribution which might explain its hyperfunctionality? To anwer these questions a study was made of the intracellular distribution of clacium and hexosamine in normal and supersensitive cat submaxillary glands *in vivo*. Both stimulated and nonstimulated glands were used in this study in an attempt to distinguish between secretion and nonsecretion processes.

EXPERIMENTAL METHODS

Thirty adult male and female cats of mixed breed were used in the study. Surgical parasympathetic decentralization of the right submaxillary gland was performed under pentobarbital anesthesia by removal of a section of the right lingual nerve after its

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juncture with the chorda-tympani nerve according to the method of Liddell and Sherrington.*[5] The left gland was unoperated and served as a normal in this study. At 10–14 days after denervation, the animals were prepared under pentobarbital anesthesia for the experiment by cannulation of the submaxillary ducts with equal lengths of Clay–Adams PE-50 tubing for the collection of saliva. Acetylcholine was administered in a volume of 0·1 ml in retrograde fashion, for 5 sec into the cannula in a dose of 0·4 μg (the usual threshold dose for the normal gland) to elicit secretion. Denervation supersensitivity was confirmed at this point by noting a 3- to 6-fold greater number of drops of saliva from the previously denervated gland. Also the femoral vein was cannulated for the administration of 12·5 μc/kg of ^{45}calcium chloride isotope. A second cannula was used for the drawing of serial blood samples. The blood samples (0·05 ml) were drawn each 15 min during the experimental period and mixed with dilute heparin (2·0 ml) and spread on the planchet for drying. The dried samples were counted in a Nuclear Chicago gas flow system of 20 per cent efficiency. Addition of a known amount of radioactivity as ^{45}calcium chloride to the blood samples followed by drying and counting revealed a high recovery of added radioactivity. Thus we feel that self-absorption of radioactivity by the blood sample is not an important consideration under these conditions.

After the demonstration of secretory supersensitivity and the administration of the ^{45}calcium isotope, both glands were stimulated to secrete in response to acetylcholine, as detailed above, each 15 min for 2 hr. Saliva from each gland was collected in one pooled sample and saved for analyses. At the end of the 2-hr period the submaxillary glands were removed and the animal was sacrificed. The glands were washed in cold 0·32 M sucrose, cleaned and then ground in cold 0·32 M sucrose in an all glass tapered homogenizer so as to prepare a 10% (w/v) homogenate. Subcellular fractions were separated by differential and gradient density centrifugation according to a modification of the procedure of Burack *et al.*[6] Eighty per cent of the total homogenate volume was centrifuged at 750 *g* for 10 min to separate the nuclei and debris. The pellet from this first spin was reconstituted with 3–4 ml of 0·32 M sucrose and recentrifuged at 450 *g* for 10 min. The supernatants from spins one and two were combined and layered over a sucrose density system. The gradient density system consisted of 5-ml portions of 2·0, 1·75, 1·5 and 1·25 M sucrose layers. This system was centrifuged in a Spinco SW-25 rotor at 24,000 rpm for 2 hr to separate the mitochondrial, microsomal, granular and debris fractions. Four distinct particulate fractions at the interfaces and a pellet resulted, which were easily separated by careful micropipette aspiration. In order to locate the mitochondria and microsomes, respectively, small aliquots of each of the subcellular fractions from the gradient density centrifugation were analyzed for the activities of succinic cytochrome C reductase and magnesium-insensitive alkaline phosphatase (see Table 1). Succinic cytochrome C reductase was assayed by a method modified from Mackler and Green in which a change in O.D/min at 550 mμ was used to calculate the micromoles of cytochrome C reduced.[7] The magnesium-insensitive alkaline phosphatase of microsomes was assayed by a modification of the procedure of Emery and Dounce with 0·1 M Tris buffer (pH 9·2), 0·2 M EDTA medium.[8] Under the conditions of this separation procedure, i.e. separation of the

* Illustrations and details of this procedure as adapted to the cat may be found in, *Studies Concerning the Relationship of Phosphorus Metabolism to Secretory Function in the Supersensitive Salivary Gland* (Thesis), H. Burford, University of Kansas (1962).

nuclei before the density gradient centrifugation, this assay serves as a useful indication of the magnesium-insensitive alkaline phosphatase of microsomes.

Chemical assays were conducted on additional aliquots from each fraction and saliva for hexosamine, protein, calcium-40 and calcium-45. The samples to be assayed for hexosamine were freed of sucrose by vacuum dialysis through collodion with a glass suction apparatus.* Negative Barfoed and Seliwanoff tests revealed that most of the sucrose had been removed by the vacuum dialysis procedure. Failure to remove traces of sucrose does not interfere with the assay of hexosamines with the *N*-acetyl modification as will be discussed later. The samples were then hydrolyzed in 2 ml of 4 N HCl for 15 hr at 100° in marble-capped tubes. The resulting solution was filtered, concentrated to 2–3 ml under vacuum and adjusted to pH 6·5 with solid NaOH in order to keep the resulting volume small. The samples were then made up to a 4·0-ml volume with distilled water. Duplicate aliquots of the above solution were assayed against glucosamine hydrochloride standard according to the acetylated amino sugar procedure of Reissig, as presented by Strominger *et al.*[9] Aliquots of all samples were treated with 10% trichloroacetic acid (TCA) to precipitate protein. After high speed centrifugation, the protein pellet was washed once with 2 ml of 5% TCA and recentrifuged. Supernatants from both centrifugations were clear and therefore were discarded. The protein pellet was solubilized in 10% sodium hydroxide for colorimetric assay. Protein was determined by the biuret[10] and the Lowry[11] methods as modified by Parvin *et al.*[12] with bovine serum albumin as a standard. Both methods were in agreement and gave values of 12–15 per cent protein by wet tissue weight. The aliquots for calcium assays were wet-digested with nitric acid and peroxide to a colorless, dry residue. The residue was solubilized with 1·5 ml of 10% nitric acid. Duplicate aliquots were assayed against calcium carbonate by the dye method of Yanagisawa as modified by Grossman and Furchgott.[13] The validity of the dye method for the estimation of calcium was confirmed by atomic absorption spectrophotometry with the model 290 Perkin–Elmer instrument. A further aliquot from the calcium digest was plated, dried and counted in a Nuclear Chicago gas flow system which gave 20–22 per cent efficiency.

Control experiments to determine the distribution of calcium-45 during homogenization and fractionation techniques were performed by adding isotope to unlabeled tissue during homogenization and maintaining conditions as outlined above. The data obtained were also used to calculate recovery percentages (see Results).

Nonstimulated experiments were performed in a fashion similar to those described above, except that no acetylcholine was administered to the animals after the surgery or during the 2-hr experimental period. In these experiments there was no statistical difference between the distribution of calcium-45 isotope under conditions *in vivo* or *in vitro*.

The data were analyzed statistically by using the Student *t*-test for paired comparison data from normal and denervated glands in the same animal and for series comparison data from stimulated and nonstimulated series of animals. Statistical analysis was performed primarily on the data in the granular fractions, since the magnitude of the differences involved and the emphasis of the study seemed to warrant this limitation.

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RESULTS AND DISCUSSION

Blood isotope

Fig. 1 gives data from a typical experiment and shows the time course of disappearance of calcium-45 isotope from the blood of the cat. At the time of i.v. injection of 12·5 μc/kg isotope, the activity of blood is in excess of 10,000 cpm. The activity decreases with time in a biphasic manner with a marked decrease in the slope of the rate line at 30 min. Our measurements were carried out for only 2 hr, at which time we saw a further decrease in the rate of disappearance, with the result that the level of isotope became fairly constant. Thus, half the calcium-45 isotope disappears from the blood of our animals in about 45 min.

These results indicate a decreasing concentration of calcium-45 isotope during our experimental period. The disappearance of plasma calcium can be explained on the basis of equilibration of this isotope with the pools of bone and soft-tissue calcium. In these experiments the effects of changes in blood flow upon calcium-45 distribution to normal vs. denervated glands are unclear. This is due primarily to a lack of information concerning the degree of vasodilatation in the denervated, supersensitive gland when acetylcholine is administered in the retrograde fashion used in this study. Emmelin *et al.* have shown for the normally innervated gland a marked increase in blood flow, persumably due to vasodilatation. Under these conditions of retrograde administration, acetylcholine causes no change in systemic blood pressure or secretion from the contralateral gland.[14] However, in this study, we observed that normal glands contain 27 per cent more calcium-45 per mg protein than denervated glands when stimulated and 32 per cent more calcium-45 per mg protein than denervated glands when unstimulated (see Table 5). Further, we note about a 40 per cent increase in calcium-45 per mg protein due to stimulation of normal and denervated gland tissue (see Table 5). This relative accumulation of calcium-45 in normal gland tissue may be related to blood flow differences or weight differences in the glands. From Table 2 we see that denervated glands weigh 26 per cent less than normal glands. However, we cannot assess the contribution of weight differences or blood flow changes on the relative accumulation of calcium-45 by the normal gland. The weight difference still exists in nonstimulated normal and denervated glands.[15]

Subcellular enzymology and microscopy profile

Table 1 gives data obtained from 10 animals by assaying for succinic cytochrome C reductase of mitochondria and magnesium-insensitive alkaline phosphatase of microsomes in all extranuclear subcellular fractions of cat submaxillary glands. Phase contrast microscopy revealed that fraction 2 contained about 90 per cent reticulum (microsomal) structures and that fraction 4 contained mitochondrial structures. Examination of fraction 3 revealed a majority of mitochondria-like structures and some microsome-like structures. The enzymology data were combined for fractions 3 and 4.

The data in Table 1 reveal the highest values for succinic cytochrome C reductase in fractions 3 plus 4, and the highest values for magnesium-insensitive alkaline phosphatase in fraction 2. Fraction 5, labeled granules, was low in enzyme activity; however, the presence of high concentrations of hexosamine served to identify this fraction from stimulated glands (see Table 4).

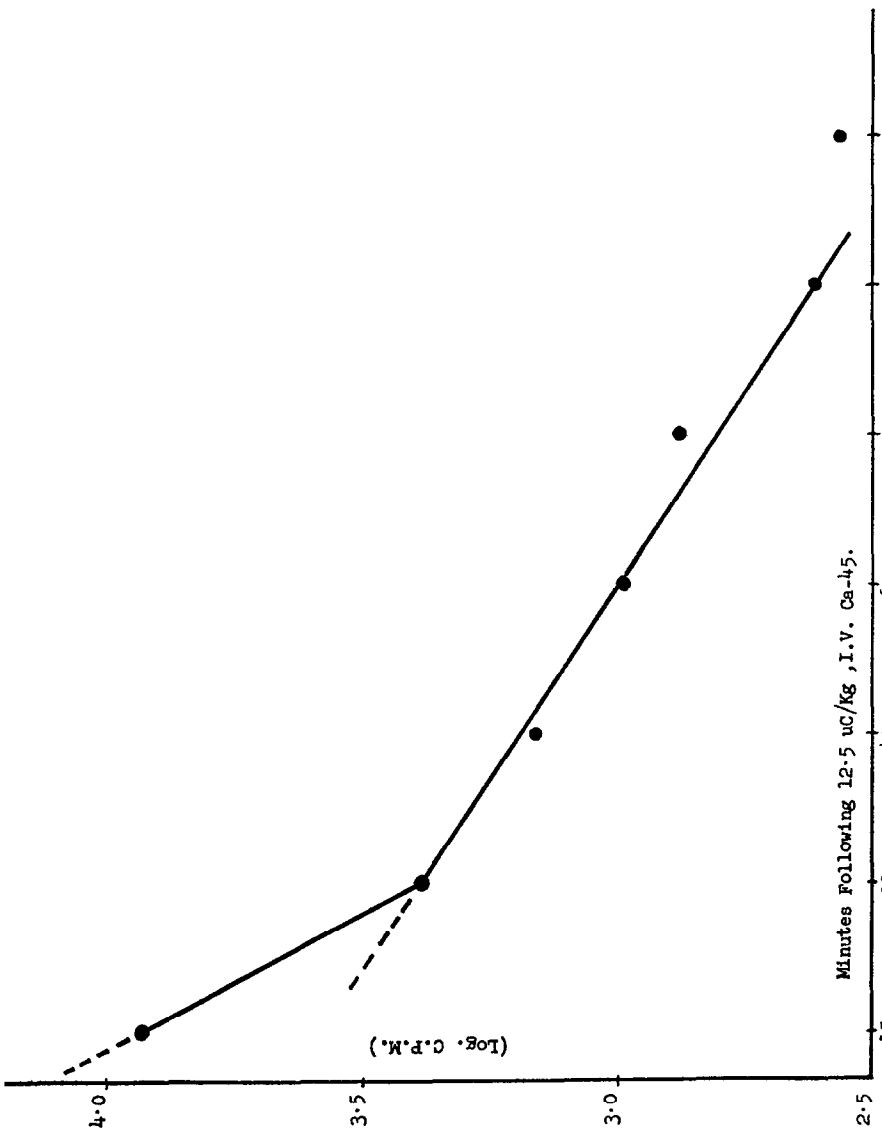

FIG. 1. Time versus calcium-45 activity in 0·05 ml blood of the cat after 12·5 μc/kg administered i.v. at zero time. Corrections made for efficiency and absorption.

TABLE 1. ENZYMOLOGY PROFILE OF SUBNUCLEAR PARTICULATE AND SOLUBLE FRACTIONS OF CAT SUBMAXILLARY GLAND*

Fraction	No.	Succinic cyt. C reductase (μmoles cyt. C reduced/min/g protein)		Mg-insensitive alkaline phosphatase (μmoles *p*-nitrophenylphosphate hydrolyzed/30 min/g protein)	
		N	D	N	D
Granules	5	0·22 ± 0·02	0·18 ± 0·01	32 ± 0·05	24 ± 0·03
Mitochondria	3 & 4	6·04 ± 0·11	5·50 ± 0·24	130 ± 1·20	121 ± 1·04
Microsomes	2	0·78 ± 0·08	0·65 ± 0·06	250 ± 1·61	212 ± 1·43
Soluble	1	0·10 ± 0·01	0·06 ± 0·01	52 ± 0·07	37 ± 0·06

* N = normal gland; D = denervated gland; number of animals = 10. Values are given ± S.E.

Control and balance data

In five experiments the average per cent recovery of added calcium-45 isotope for normal glands was 108 ± 5 (S.E) while the average recovery for denervated glands was 90 ± 6 (S.E.).

The isotope data from the homogenization and fractionation control experiments expressed as per cent of the total isotope found in each fraction revealed that calcium-45 *in vitro* distributed itself very much as it did when injected i.v. (in stimulated glands), with the notable exception of the mitochondrial and granular fractions, which contained significantly more isotope under experimental than under control conditions. These experiments on stimulated normal and denervated glands revealed that the nuclear fraction contained 40–45 per cent of the total isotope, the supernatant fraction contained 35–40 per cent of the total isotope and the microsomal fraction contained 12–13 per cent of the total isotope. This was true whether the isotope was administered *in vitro* (control) or *in vivo* (experimental). The total per cent isotope contained in these three fractions was from 87 to 90 per cent. The small per cent of isotope remaining was distributed between the mitochondria and the granules in the following manner. Under conditions *in vivo*, the mitochondria from normal stimulated glands concentrate 1·5 times more isotope and mitochondria from denervated stimulated glands concentrate 2·0 times more isotope than mitochondria under conditions *in vitro*. Similarly, granules from stimulated glands under conditions *in vivo* concentrate 3·0 times more isotope in the normal gland and 2·0 times more isotope in the denervated gland than granules under conditions *in vitro*. The data *in vivo* (experimental) in Tables 4 and 5 were corrected for the difference in calcium distribution in the mitochondrial and granular calcium-40 and calcium-45 by the amount of calcium isotope which accumulated under conditions *in vitro*. The seeming lack of significance between the data *in vitro* (control) and *in vivo* (experimental) for calcium distribution to mitochondria and granules may perhaps be explained by the relatively small percentage of calcium which is distributed to these fractions in relation to the other subcellular fractions.

Our calcium-40 balance studies conducted on all glands studied revealed that we were able to recover in the fractions 100 ± 20 per cent of all calcium found in the total homogenate. The balance data for hexosamines were of the same magnitude. However, the balance data for protein were less satisfactory. The average amount in the subcellular fractions was only 68·2 ± 1·7 per cent of that found in the total homogenates (see Table 3). Since the balance data for protein are lower than we had anticipated,

this raises the possibility that the protein content of the granular fraction especially may be falsely low. For this reason we shall report total amounts of hexosamine and calcium in the granule fractions from all glands in addition to the concentration data in Tables 4 and 5.

Salivary data

Table 2 presents the saliva data from the series of 20 animals stimulated with acetylcholine. The decrease in gland weight seen after denervation is barely significant.

TABLE 2. SALIVA DATA (ACETYLCHOLINE STIMULATION)*

Gland type	Gland wt. (g)	Volume saliva (ml)	Hexosamine (μg/ml)	Calcium (μg/ml)	Calcium-45 (cpm/ml)	Sp. act. (cpm/μg Ca)
Normal	1·75 ± 0·2	0·56 ± 0·1	425 ± 44·7	43 ± 5·4	1466 ± 201	34·0
Denervated	1·29 ± 0·1	1·62 ± 0·2	515 ± 68·0	49 ± 4·7	1629 ± 220	33·0

* No. of animals = 20. Values are given ± S.E.

However, the increased saliva volume is significant and is typical of denervation supersensitivity. In this series of animals there is no increase in the concentration of hexosamine or calcium in the saliva and denervated glands. However, since the denervated gland secretes some 3 times more saliva than the normal gland, the total output of hexosamine and calcium is correspondingly greater and thus the total output values are significantly different ($P < 0·01$).

The secretion of calcium has been studied, particularly in the dog, and values of 4–6 mEquiv/l. in submaxillary saliva after chorda stimulation have been reported by Kesztyüs and Martin.[16] This work also noted that the concentration was practically independent of the rate of salivary flow. Also, the secretion of calcium by the submandibular gland of the rat has been studied by Driesbach.[17] He reports 1·7 μmole/ml calcium-40 at 5 min after pilocarpine maximal stimulation and 0·5 μmole/ml calcium-40 at 1 hr after pilocarpine in the same animal. Our data show values of 1·01 μmole/ml calcium-40 for saliva from the normal gland and 1·22 μmole/ml calcium-40 for saliva from the denervated gland. These saliva samples were pooled during the course of the experiment and are very near an average of the values reported by Driesbach. However, the secretion of calcium, especially in the supersensitive gland of the cat has not been studied. It has been shown that more sodium is secreted from the supersensitive gland than from the normal gland in response to both acetylcholine and epinephrine.[15]

By calculation from data presented in Table 2, we see that the molar ratio of hexosamine to calcium secreted in saliva is approximately 2·3 for both the normal (N) and the denervated (D) glands. (N = 2·4 μM hexosamine/ml ÷ 1·06 μM Ca/ml = 2·26 and D = 2·9 μM hexosamine/ml ÷ 1·22 μM Ca/ml = 2·38.) As we shall see in Table 4, this ratio of hexosamine to calcium is less than that found in the normal, stimulated gland granular fraction (3·7). These results will be discussed in the sections dealing with hexosamine and calcium.

Subcellular distribution data

Protein. Table 3 presents the protein data for stimulated (S) and nonstimulated (NS)

cat submaxillary glands in this study. Calculation of average per cent protein by wet weight from the total homogenate average values reveals the following for each type of gland: 11·0 ± 2·4 per cent for NS, 14·9 ± 3·5 per cent for DS, 15·4 ± 1·0 per cent for NNS and 17·8 ± 2·1 per cent for DNS. In the footnote to Table 3, the *t*-test reveals that the granular fraction from nonstimulated glands contains a significantly greater percentage protein than the stimulated glands in spite of rather large standard errors. The P value for the series comparison between DNS and DS $< 0{\cdot}001$, while the P value for comparison between NNS and NS $< 0{\cdot}01$.

TABLE 3. PROTEIN DATA (TOTAL mg PROTEIN—BIURET ± S.E.)

Cell fraction		No. of animals = 20		No. of animals = 10	
		Normal glands stimulated (NS) (1·75 ± 0·2)*	Denervated glands stimulated (DS) (1·29 ± 0·1)*	Normal glands nonstimulated (NNS) (1·33 ± 0·1)*	Denervated glands nonstimulated (DNS) (1·09 ± 0·2)*
Total homogenate		193·8 ± 16·5	191·6 ± 18·5	204·9 ± 14·6	194·7 ± 11·7
Nuclei		82·7 ± 6·5	63·6 ± 5·4	87·4 ± 6·6	68·3 ± 4·7
Cell sap	1	33·6 ± 2·9	22·8 ± 2·0	43·7 ± 6·6	35·1 ± 3·0
Microsomes	2	4·5 ± 0·3	5·0 ± 0·7	8·2 ± 0·6	5·7 ± 1·6
Mixed	3	2·3 ± 0·3	2·7 ± 0·4	3·5 ± 0·8	2·8 ± 0·8
Mitochondria	4	1·9 ± 0·06	1·7 ± 0·2	2·6 ± 0·5	1·8 ± 0·3
Granules†	5	0·59 ± 0·01	0·64 ± 0·01	1·8 ± 0·03	1·8 ± 0·02
Debris	6	10·3 ± 0·4	11·8 ± 1·3	13·1 ± 3·5	17·9 ± 3·2

* Average gland weights (g wet) ± S.E.
† Granules: NS/S, normal = 3·05, denervated = 2·81; P = < 0·001.

This decreased protein in the stimulated gland is most evident in the microsomal (NS = 1·8 × S) and granular (NS = 3·0 × S) fractions for normal glands and in the granular fraction for denervated glands (see Table 3, bottom). The decrease in protein in the granular fraction of stimulated glands probably reflects loss of granular protein by the stimulated gland during secretion.

Hexosamine. Table 4 (upper part) presents the hexosamine concentrations in the glands studied, expressed as micromoles of hexosamine per gram of protein. Hexosamine represents salivary mucin, being derived from it by acid hydrolysis. The most striking things about the data are the following. (1) Both the normal and denervated stimulated glands have a higher hexosamine concentration in the granular fraction than do nonstimulated glands. The normal glands contain more hexosamine than do the denervated ones. The ratio of NS/DS for hexosamine in the granular fraction is 2·61, with a P value of 0·05 to 0·01. No other fraction contains this much more hexosamine in the normal gland. The nuclei, mixed and mitochondrial fractions contain about 1·3 times more hexosamine in the normal gland. (2) In nonstimulated glands the concentration of hexosamine in the granular fraction is not so marked as in the stimulated glands. In fact, the granules contain no more hexosamine than the mitochondrial fraction per gram of protein. 3) The difference in hexosamine content of granules from stimulated and nonstimulated glands is most marked. The ratio of S (stimulated) to NS (nonstimulated) shows that normal glands accumulate 13·9 times more hexosamine in their granules when stimulated than when nonstimulated, and further, that denervated glands accumulate 6·4 times more hexosamine in their

TABLE 4. HEXOSAMINE AND CALCIUM DATA*

Cell fraction	No. of animals = 20				No. of animals = 10			
	Normal glands stimulated (NS)	Hxam/Ca	Denervated glands stimulated (DS)	Hxam/Ca	Normal glands nonstimulated (NNS)	Hxam/Ca	Denervated glands nonstimulated (DNS)	Hxam/Ca
Hexosamine								
Total homogenate	181 ± 5·6		214 ± 6·9		136 ± 7·9		124 ± 6·6	
Nuclei + debris	390 ± 11·6	1·4	287 ± 5·4	1·1	222 ± 5·4	2·0	182 ± 4·5	1·6
Cell sap	379 ± 16·8	2·1	578 ± 14·1	3·7	129 ± 2·5	1·8	172 ± 3·8	1·9
Microsomes	390 ± 18·1	1·2	598 ± 11·6	2·5	199 ± 4·7	1·4	191 ± 5·4	1·0
Mixed	562 ± 20·6	1·6	420 ± 6·5	1·6	246 ± 8·5	0·9	294 ± 9·8	1·2
Mitochondria	1258 ± 39·1	1·5	998 ± 16·6	1·4	373 ± 13·3	2·0	317 ± 8·5	1·8
Granules	5300 ± 106	3·7	2025 ± 53·7	2·1	379 ± 11·4	1·7	319 ± 9·2	0·9
Calcium								
Total homogenate	98 ± 3·4		80 ± 2·9		60 ± 3·5		65 ± 2·8	
Nuclei + debris	280 ± 11·4		258 ± 4·9		112 ± 2·5		115 ± 5·1	
Cell sap	180 ± 6·0		157 ± 4·3		73 ± 2·2		93 ± 1·9	
Microsomes	318 ± 14·3		240 ± 5·8		145 ± 5·4		178 ± 8·5	
Mixed	360 ± 17·9		258 ± 7·6		285 ± 3·8		240 ± 3·8	
Mitochondria†	822 ± 21·7		736 ± 12·3		185 ± 8·2		173 ± 5·1	
Granules†	1420 ± 33·6		965 ± 14·3		218 ± 6·0		363 ± 8·5	

* Hxam = hexosamine. Values are given as μequiv/g protein ± S.E.
† Correct π values; see text, Control and balance data.

granules when stimulated. Both differences are significant (P = 0·001). The total amount of hexosamine, neglecting the concentration of protein, in the granule fractions of the glands studied are calculated from Tables 4 and 2 as follows: NS = 560 μg, DS = 232 μg, NNS = 122 μg and DNS = 103 μg hexosamine. Further discussion of hexosamine will be reserved for the subsequent section on calcium-40.

Calcium-40. Table 4 (lower part) also presents the total calcium data in the glands studied, expressed as microatoms of calcium per gram of protein. We see an accumulation of calcium per gram of protein in the stimulated gland, particularly in the mitochondrial and granular fractions. However, unlike granular hexosamine in which the normal stimulated gland contained more than the denervated stimulated gland, granular calcium did not accumulate to a significantly greater extent in the normal gland (NS/DS = 1·47, P = 0·5 to 0·1). Also, as seen with hexosamine, the stimulated gland accumulated more calcium in the granular fraction than the nonstimulated gland. The ratio S/NS for the granular fraction of normal gland is 6·5 and for denervated glands is 2·6 with P values of 0·001. The total amount of calcium, neglecting the concentration of protein, in the granule fractions of the glands studied is calculated from Tables 4 and 2 as follows: NS = 33·5 μg, DS = 24·7 μg, NNS = 15·7 μg and DNS = 26·1 μg calcium.

In order to obtain some idea of the relative amounts of hexosamine and calcium in each fraction, we have calculated the following data from Table 4.

In Table 4 we see 1420 microatoms calcium/g protein in the normal stimulated granular fraction. This is some 14·5 times the calcium concentration in the total homogenate (98 microatoms/g protein). The concentration of hexosamine (5300 μmole/g protein) in the normal stimulated gland is some 29 times higher in the granular fraction than in the total homogenate of the normal stimulated cells (181 μmole/g protein). Also, the ratios of μmole/ of hexosamine to microatoms of calcium present in the various fractions of the glands studied are calculated from the data and presented in Table 4 (upper part). The fact that the normal stimulated cell sap and microsomal fractions have a higher hexosamine to calcium ratio than the granular fraction of stimulated glands is due to a relatively lesser degree of concentration of calcium in the former two fractions rather than to a greater relative concentration of hexosamine. Preliminary experiments in our laboratory indicate that this observation in the denervated gland especially may be explained by a temperature-dependent leakage of mucin from secretory granules, much like the leakage of amylase from the zymogen granules of rat parotid gland at cold temperatures (0°) reported by Schramm *et al.*[18] The ratios of hexosamine to calcium for the nonstimulated glands reveal that although the granular fractions are highest in hexosamine, the calcium content of the granular fraction is also relatively high, thus lowering the ratio for normal and denervated glands (Table 4).

It should be pointed out that the data in Table 4 are concentration based on the content of protein. For example, the granule fraction contains the least protein (Table 3) but is highest in concentration of hexosamine/g protein. The total values for granular hexosamine and calcium-40 are calculated from the data in Tables 2 and 4 to neglect the concentration of protein and are reported in the appropriate sections of the Discussion (above).

Calcium-45. Table 5 presents calcium-45 activity data from the glands used in this study expressed as cpm/mg protein. As with the total calcium "pool", there is

TABLE 5. CALCIUM-45 DATA*

Cell fractions	No. of animals = 20 Normal glands stimulated (NS)	Denervated glands stimulated (DS)	No. of animals = 10 Normal glands nonstimulated (NNS)	Denervated glan nonstimulated (DNS)
Total homogenate	63·2 ± 2·7	46·2 ± 1·8	37·4 ± 0·9	25·5 ± 1·3
Nuclei	36·9 ± 0·9	27·8 ± 0·8	29·6 ± 2·2	27·0 ± 0·9
Cell sap	71·7 ± 2·2	85·6 ± 2·7	40·8 ± 2·8	38·1 ± 1·9
Microsomes	79·4 ± 2·7	90·0 ± 3·1	67·1 ± 3·5	75·1 ± 3·8
Mixed	110·9 ± 3·1	72·4 ± 2·0	59·3 ± 3·2	83·3 ± 5·4
Mitochondria†	270·0 ± 4·7	161·9 ± 3·4	44·4 ± 2·5	42·8 ± 2·2
Granules†	487·0 ± 22·8	241·0 ± 12·9	45·9 ± 1·5	44·2 ± 2·8
Debris	528·0 ± 13·4	38·5 ± 1·8	19·8 ± 1·9	11·6 ± 2·5
Sp. act. (cpm/μg Ca)				
		NS DS	NNS DNS	
Total homogenate		16·2 14·4	15·6 9·8	
Granules‡		8·6 6·2	5·3 3·1	

* Values are given as cpm/mg protein ± S.E.

† See footnote, Table 4.

‡ Granules: S/NS, normal = 10·6, denervated = 5·45, P = 0·001; NS/DS = 2·03, P = 0·05 to 0·1.

concentration of activity in the granular fraction. The values for granule calcium are only significant, however, when one compares the stimulated (S) to the nonstimulated (NS) glands (Table 5, footnote). Also we again see that the normal, stimulated gland can concentrate granular calcium slightly better than the denervated stimulated gland. The sp. act. of the calcium "pool" for the total homogenate and granular fraction is also listed on Table 5. The granular calcium shows a progressive increase as the effect of stimulation is imposed on the denervated nonstimulated gland. The data with calcium isotope merely reveal what is happening in the total calcium pool.

From the data in Tables 2, 3 and 4, the answers to the problems posed in this work can be discussed. It is obvious from the data in Table 4 that the hexosamine content in stimulated glands is significantly higher than that in nonstimulated glands. The P value for the granular fraction alone is less than 0·001 for the difference in hexosamine content. Also the content of calcium is significantly greater in stimulated than in nonstimulated glands. The value is $P < 0{\cdot}001$ for the granular fractions involved as already pointed out. Since more hexosamine and calcium were secreted from the stimulated glands (Table 2), these results indicate that stimulation leads to accelerated hexosamine synthesis and increased uptake of calcium into the gland. The magnitude of these increases in hexosamine and calcium content appear rather striking in view of the fact that the glands were secreting for only about 30 min of the 2-hr experimental period.

The hexosamine concentration of the granule fraction was the largest of any fraction measured at the end of the experimental period in stimulated glands, despite a reduction in protein content. Although not definite, these results suggests that hexosamine-rich granules are being formed or restored after the secretory stimulus and that the bulk of the protein is not as rapidly formed.

Also, the denervated glands secreted about 3·5 times more hexosamine (834 μg, total) than did the normal glands (238 μg, total; Table 2). In the absence of synthesis, the denervated gland granules would thus be expected to contain less hexosamine than

normal glands. This is the case for the granules of nonstimulated glands (Table 4), although one would expect some degree of synthesis in nonstimulated glands. By comparing stimulated and nonstimulated glands we find an increase in total granule hexosamine: normal from 682 to 3127 μmole hexosamine and denervated from 574 to 2295 μmole hexosamine, total (correcting for protein content of the granule fraction). This indicates an excess production of granule hexosamine which is relatively greater for the normal glands due to stimulation. Either the effects of denervation *per se* or the greater loss of cellular material during stimulation may have contributed to the relatively smaller excess production of granule hexosamine by denervated glands.

Therefore we feel that the data presented may implicate calcium accumulation in the synthesis of hexosamine or its storage in the granular fraction. Also, we can find nothing distinctive about the manner in which the denervated gland handles calcium that might explain its supersensitive nature.

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