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A QUALITATIVE AND QUANTITATIVE HISTOCHEMICAL INVESTIGATION OF THE EFFECTS OF PILOCARPINE STIMULATION ON AMYLASE LEVELS IN RAT SUBMAXILLARY AND PAROTID GLANDS[1]

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A histochemical study, using the starch substrate film technique, was done to compare amylase activity in fresh-frozen cryostat sections of paired male rat parotid and submaxillary-sublingual glands, before and after pilocarpine stimulation. A quantitative procedure was followed on the same material. Amylase depletion was apparent in sections of parotid gland incubated on starch films for short periods. Quantitative estimations on adjacent sections showed an amylase depletion of about 50%. In the submaxillary-sublingual gland, amylase was demonstrable in the blood vessels in both unstimulated and stimulated material and there appeared to be a higher concentration of the enzyme in the latter. Quantitative assays on adjacent sections demonstrated a very considerably raised level of amylase activity in the stimulated submaxillary gland. It was concluded that the histochemically demonstrable amylase in the blood vessels of the submaxillary and sublingual glands was blood amylase and that the raised level following pilocarpine stimulation might be interpreted as a passage to the blood of enzymes produced by other glands under the same stimulus.

It was shown by Schneyer and Schneyer (5) that in the fasting rat stimulation by feeding or by injecting pilocarpine depleted parotid gland amylase by up to 50% but that similar stimulation resulted in a very considerable increase in amylase levels (as much as 16-fold) in the submaxillary-sublingual gland complex. This phenomenon can be blocked by atropine.

Three possible mechanisms were proposed to account for this increased amylase level in the submaxillary gland complex: (*a*) activation of a proenzyme or a "masked" enzyme, (*b*) increased serum space or serum amylase level and (*c*) synthesis of new enzyme by submaxillary-sublingual gland tissue cells. Schneyer and Schneyer (5) believed that the phenomenon could best be explained by assuming a synthesis of new enzyme under the control of the parasympathetic nervous system.

Sreebny and Meyer (9) and Junqueira, Toledo and Saad (2) contended, however, that the observed increase of submaxillary gland amylase results from an accumulation of blood amylase in the gland. Another interpretation was proposed by Chignell (1). His experiments showed that the incorporation of ^{14}C-labeled amino acids into rat submaxillary gland amylase decreased after the administration of pilocarpine and that, furthermore, both actinomycin D and puromycin failed to block the pilocarpine-stimulated increase in rat submaxillary gland enzyme levels. Chignell felt that these results suggested that pilocarpine does not stimulate the *de novo* synthesis of rat submaxillary gland amylase. His data showed that there was a potentiation of the pilocarpine effect at a high dose level of actinomycin D and suggested that this probably results from inhibition of *de novo* synthesis of catabolic enzymes. He postulated therefore that if, under the influence of pilocarpine, the rate of amylase catabolism decreased while the rate of synthesis remained the same, then the enzyme level would rise.

Recently it was shown (6) that, using a 3% starch substrate film technique, amylase activity in the rat submaxillary-sublingual complex could be localized in the large interlobular arterioles, in the vessels associated with the larger intralobular ducts and also in the fine capillaries that intertwine extensively between the secretory acini and around the granular convoluted tubules. This observation led us to the study reported in the present paper, the

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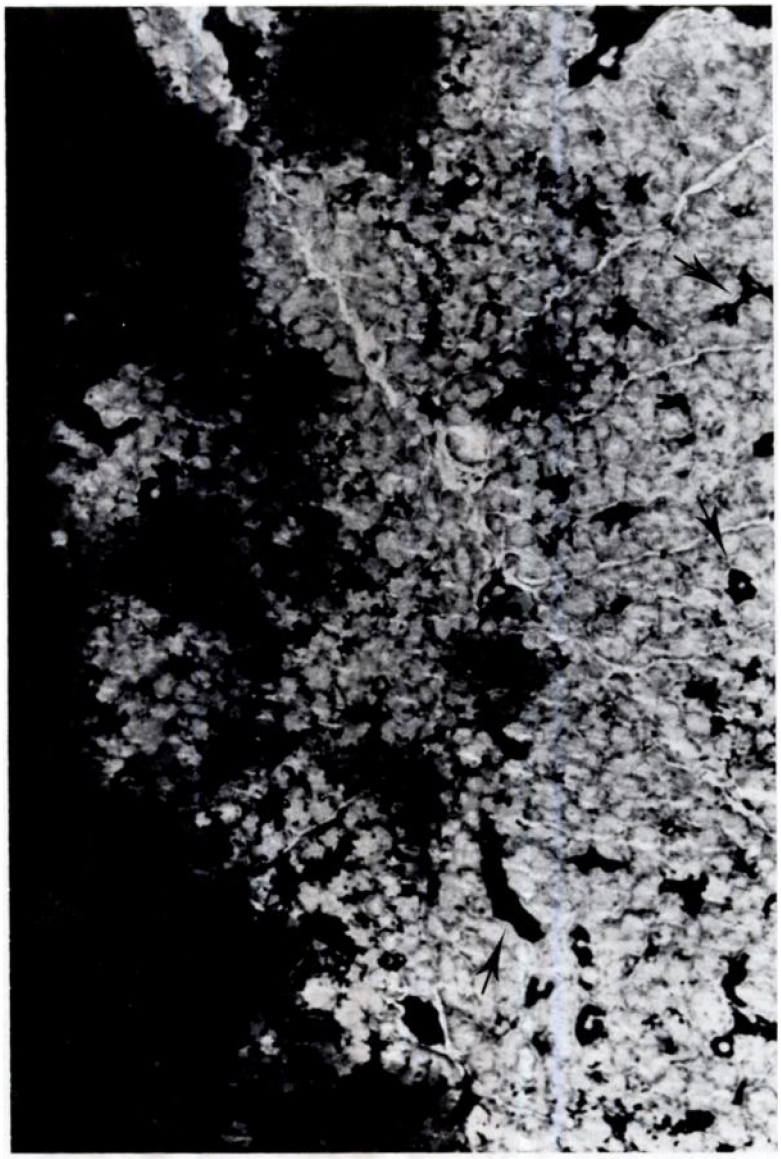


FIG. 1. Fresh-frozen cryostat section of fasting adult male rat parotid gland incubated on a 5% starch film for 1 min. The clear areas indicate amylase activity; the dark areas represent undigested periodic acid-Schiff-stained starch film. Extensive amylase activity is seen on the right side of the photomicrograph where only the duct lining cells (some of which have been indicated by arrows) are negative. Starch-PAS. ×100.

main purpose of which was to test, by histochemical means, the hypothesis that the considerable rise in amylase levels in rat submaxillary gland following pilocarpine stimulation results from an increased level of this enzyme in the blood. At the same time we sought to demonstrate histochemically the effects of pilocarpine stimulation on rat parotid amylase activity.

MATERIALS AND METHODS

The experiments were done on the submaxillary-sublingual glands of seven adult male Wistar strain

rats, ranging in weight from 270 to 390 g (mean 343 g), and on the parotid glands of six of these.

In each case the animal was fasted overnight but allowed water. The next morning it was anesthetized with Thalamonal (0.25 ml/100 g body weight intramuscularly). The submaxillary and parotid glands on the right side were removed for control studies and quenched in liquid nitrogen. The wound was sutured and the animal returned to its cage and fed. That night the animal was again fasted and the next morning was given 18 mg pilocarpine nitrate subcutaneously in doses of 2 mg at ½-hr intervals. Each 2-mg dose was dissolved in 0.4 ml deionized water (0.5% solution). About 15 min after the last injection, the animal was killed with coal gas and the glands on the left side were rapidly dissected and quenched in liquid nitrogen.

Control and experimental glands were subjected to



FIG. 2. Comparable section of rat parotid gland following pilocarpine stimulation. Section incubated on 5% starch film for 1 min. Amylase activity is less extensive and can be localized (clear areas) to individual acini and duct lumina. Starch-PAS. ×100.



FIG. 3. Higher magnification of part of Figure 2 showing cellular localization of amylase activity. Starch-PAS. × 250.

the same procedures. For the qualitative studies substrate films made of 3 and 5% solutions of hydrolyzed starch were used (6, 7). Fresh-frozen cryostat sections were cut at 8-12 μm and mounted directly on the starch films. Sections of submaxillary gland were mounted on 3 and 5% films. Sections of parotid were mounted on 5% films. After incubation in a moist chamber at 37°C for periods ranging from 1 to 30 min, corresponding to the incubation times used in the quantitative studies (Figs. 4 and 11), the reaction was stopped and the sections were fixed by immersing the preparations in a solution of acetic acid-methanol. After washing, the starch films were stained by the periodic acid-Schiff (PAS) method and nuclei were counterstained with hematoxylin. The preparations were then dehydrated, cleared and mounted in DPX.

For the quantitative studies, cryostat sections were cut serial to those used for the qualitative work. For

the parotid gland investigations, 14 consecutive sections were cut and each one was collected separately in a cold 6.5- x 2.5-cm plastic tube with screw top (6). For the submaxillary gland investigations, 70 consecutive sections were cut and 7 were collected in each of 10 cold plastic tubes. These sections were used for amylase assays. For both parotid and submaxillary studies, 4 other tubes were used into which were harvested 5, 7, 8 and 10 sections, respectively. These were kept frozen and used for protein assays. All of the foregoing sections were cut within ½ hr so that water loss from the specimen was reduced to a minimum.

Amylase was estimated using an adaptation of the method of Smith and Stocker (8) and the protein content was estimated according to the method of Lowry *et al.* (4). Details of the procedures followed are described elsewhere (6). Amylase activity was calculated as follows:

(mg maltose formed/section)/(μg protein/section)

and the quotient was plotted against time.

The significance of the differences in activity between unstimulated and stimulated glands was calculated using Student's *t*-test (3).

The black and white photomicrographs were photographed through a green filter using Ilford FP4 film and printed on glossy, single weight Ilfrobrom 1B2 1P.

RESULTS

Parotid: The unstimulated parotid shows fairly extensive digestion of the starch films after short periods of incubation. The high amylase content diffuses rapidly and intracellular localization becomes very difficult to demonstrate (Fig. 1). With the stimulated parotid incubated for 1 min, large areas of undigested film are present and intracellular localization is possible (Figs. 2 and 3).

The results of the quantitative studies on unstimulated and stimulated parotids are shown in Figure 4.

Amylase activity is read at 5 min and is expressed as milligrams of maltose liberated per microgram of protein in 5 min at 37°C. This gives a mean reading of amylase activity in six rats of 0.302 ± 0.084 (S.D.) for unstimulated parotid and 0.152 ± 0.061 for stimulated parotid. This difference is statistically significant (t = 6.02; $P < 0.2\%$).

Submaxillary-sublingual complex: There is no histochemical evidence of amylase activity in sections of unstimulated submaxillary-sublingual gland incubated on 5% starch films for periods of up to 30 min. Figure 5 illustrates such an unstimulated submaxillary gland after 10 min of incubation. No digestion of the starch film has taken place. In the stimulated gland, however, sections incubated for 10 min on a 5% film do show digestion, and the amylase activity is localized to the blood vessels (Fig. 6). The most satisfactory localization, however, is demonstrable when sections are incubated on 3% films. Figure 7A and B illustrates consecutive sections of the same area of submaxillary gland incubated on a 3% starch film for 7 sec. Amylase activity is seen in the larger vessels surrounding the excretory ducts and also in the fine capillaries which intertwine around the granular tubules and the secretory acini. The acinar cells

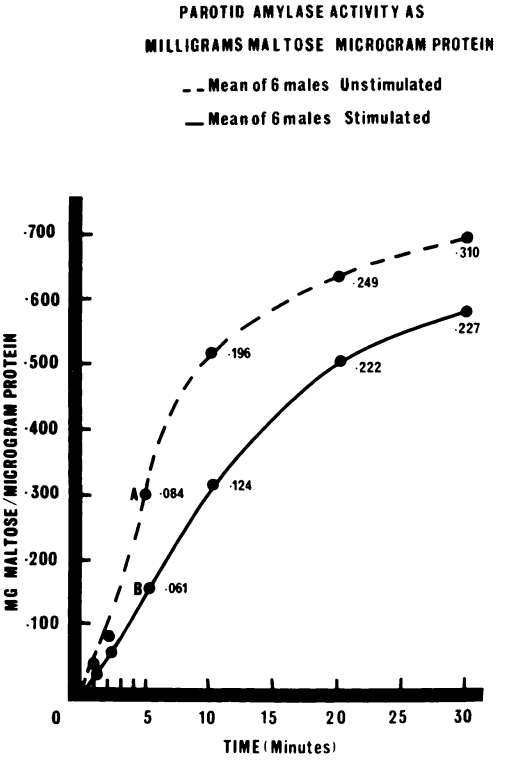


FIG. 4. Rat parotid amylase activity in fasting and pilocarpine-stimulated animals. Activity is read at 5 min as milligrams of maltose per microgram of protein at 37°C. Mean amylase activity in unstimulated parotids of six rats is 0.302 ± 0.084 (S.D.) and 0.152 ± 0.061 in stimulated parotids (points A and B, respectively). This difference is statistically significant (t = 6.02; $P < 0.2\%$). Figures shown at points along the curves are standard deviations.



FIG. 5. Section of submaxillary gland of fasting adult male rat incubated on 5% starch film for 10 min. There is no histochemical evidence of amylase activity. Starch-PAS. · 250.

themselves appear to be negative and there is no demonstrable activity in the lumina of the granular tubules or excretory ducts. The localization in Figure 7A is virtually identical with that in Figure 7B, and this tends to verify its accuracy.

When sections of stimulated submaxillary are incubated on 3% films for periods of 10 or 20 min, there may be considerable diffusion of amylase (Figs. 8 and 9). In the sublingual gland, amylase is also localized to the blood vessels (Fig. 10).

The results of the quantitative studies on unstimulated and stimulated submaxillary-sublingual are shown in Figure 11. In Figure 11, the scale on the ordinate axis is $\frac{1}{100}$ that used to

chart amylase activity in the parotid. The unstimulated submaxillary shows negligible activity over an incubation period of 30 min. The activity in the stimulated glands, however, increases linearly over this period. After 10 min of incubation, sections of unstimulated gland show a mean amylase activity of 0.00016 ± 0.00000 (S.D.) mg maltose/μg protein, whereas stimulated tissue shows an activity of 0.0016 ± 0.0010. This difference is statistically significant (t = 3.80; $P < 1\%$). After 30 min the numerical differences are still greater: 0.00024

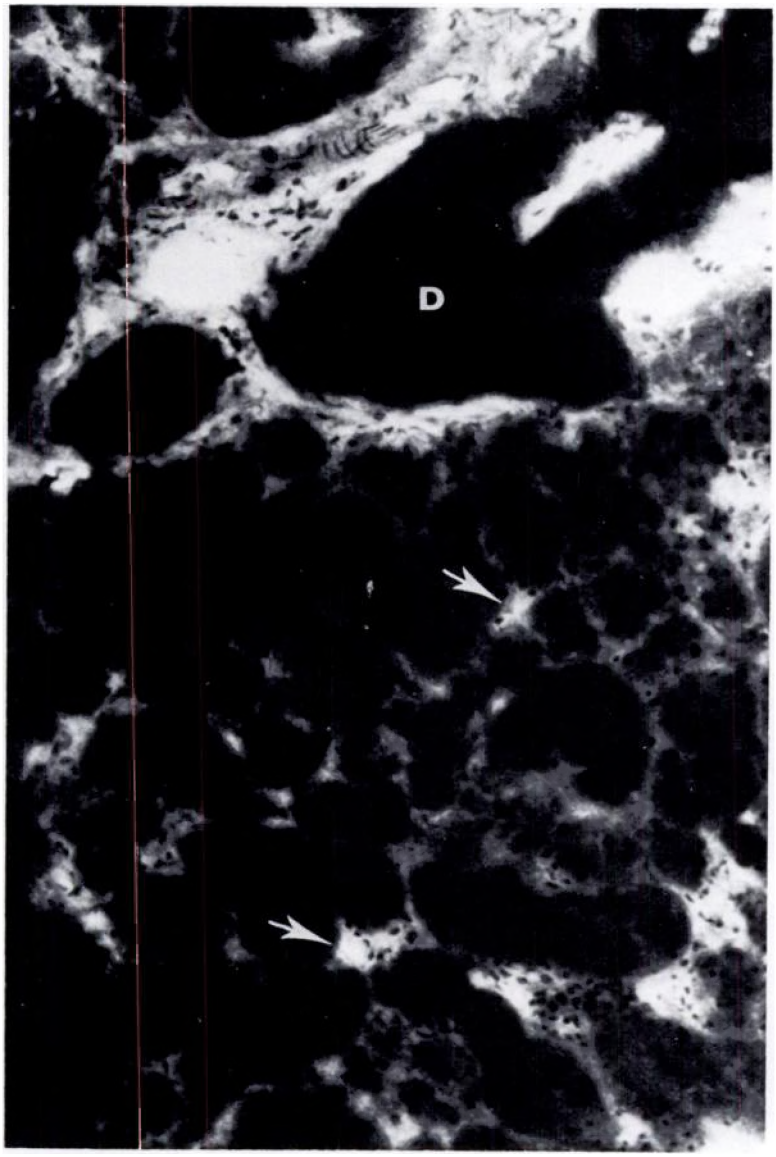


FIG. 6. Section of submaxillary gland of adult male rat after pilocarpine stimulation and incubated on 5% starch film for 10 min. Clear areas, indicative of amylase activity, are seen in the blood vessels surrounding the large interlobular duct (D) and also in the fine capillaries (indicated by arrows) between the secretory acini. Note that the lumen of duct D and the secretory acini themselves are negative for amylase. Starch-PAS. ×250.

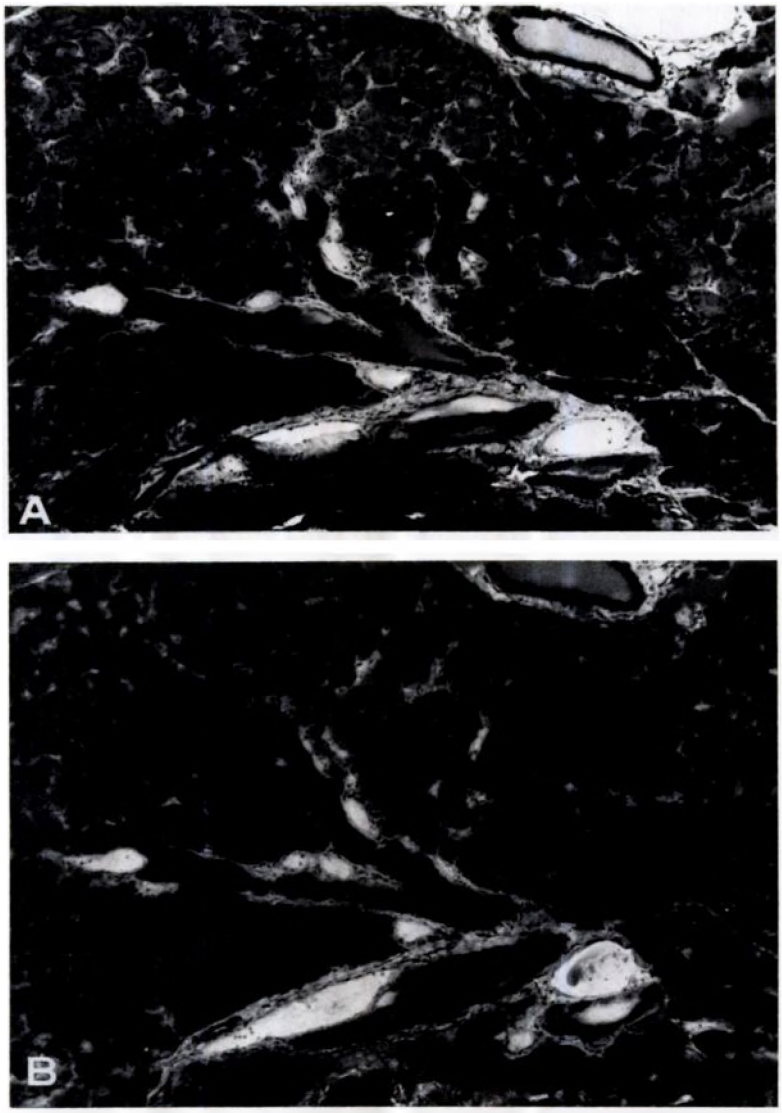


FIG. 7. A and B, photomicrographs of the same field in serial sections of fasting rat submaxillary gland mounted on 3% starch film and incubated for 7 min. Localization of amylase activity is seen in the same blood vessels in both sections. Secretory acini and contents of duct lumina are negative. Starch-PAS. ×50.

± 0.00000 for unstimulated and 0.0044 ± 0.0032 for stimulated tissue. This difference is statistically significant (t = 3.44; P < 2%).

The protein content in the unstimulated parotids of six rats ranged from 12.5 to 51.1 μg with a mean of 30.2 ± 13.1 (S.D.), and in the stimulated parotids it ranged from 15.3 to 34.5 μg with a mean of 23.2 ± 6.4. This difference is not statistically significant (t = 1.31; P > 10%). The protein content in the unstimulated submaxillary glands of seven rats ranged from 89.7 to 144.4 μg with a mean of 119.9 ± 18.2, and in the stimulated submaxillary glands the range was from 70.7 to 110.7 μg with a mean of 87.6 ± 13.4. This difference is statistically significant (t = 6.36; P < 0.2%).

DISCUSSION

The quantitative histochemical studies confirm the biochemical findings of a number of

workers (1, 2, 5) that pilocarpine stimulation reduces the amylase activity in rat parotid by about 50% and produces a very considerable rise in amylase content of the submaxillary-sublingual. The amylase depletion in the parotid can be demonstrated using the starch substrate film technique when short periods of incubation (not more than 1 min) are employed. Even after prolonged pilocarpine stimulation, there is still a very high amylase content in the secretory acini of rat parotid and this rapidly digests the thin starch film.

Qualitative studies of the submaxillary-sublingual gland show convincingly that virtually all its histochemically demonstrable amylase lies within blood vessels and is therefore blood

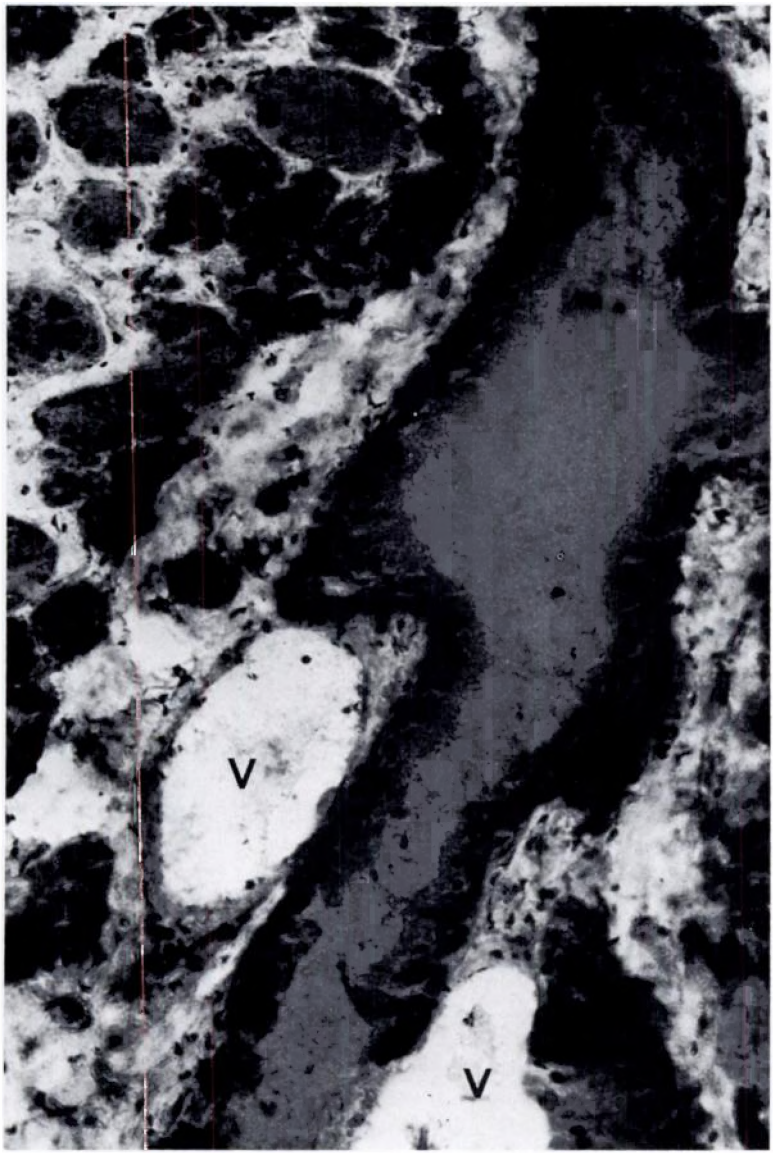


FIG. 8. Section of submaxillary gland of rat after pilocarpine stimulation, incubated on 3% film for 10 min. The contents of the larger blood vessels (V) are positive for amylase, as are the fine interacinar capillaries. There appears to have been diffusion of enzyme, so that accurate localization to the vessels is not seen. The contents of the large excretory duct are negative. Starch-PAS. · 250.

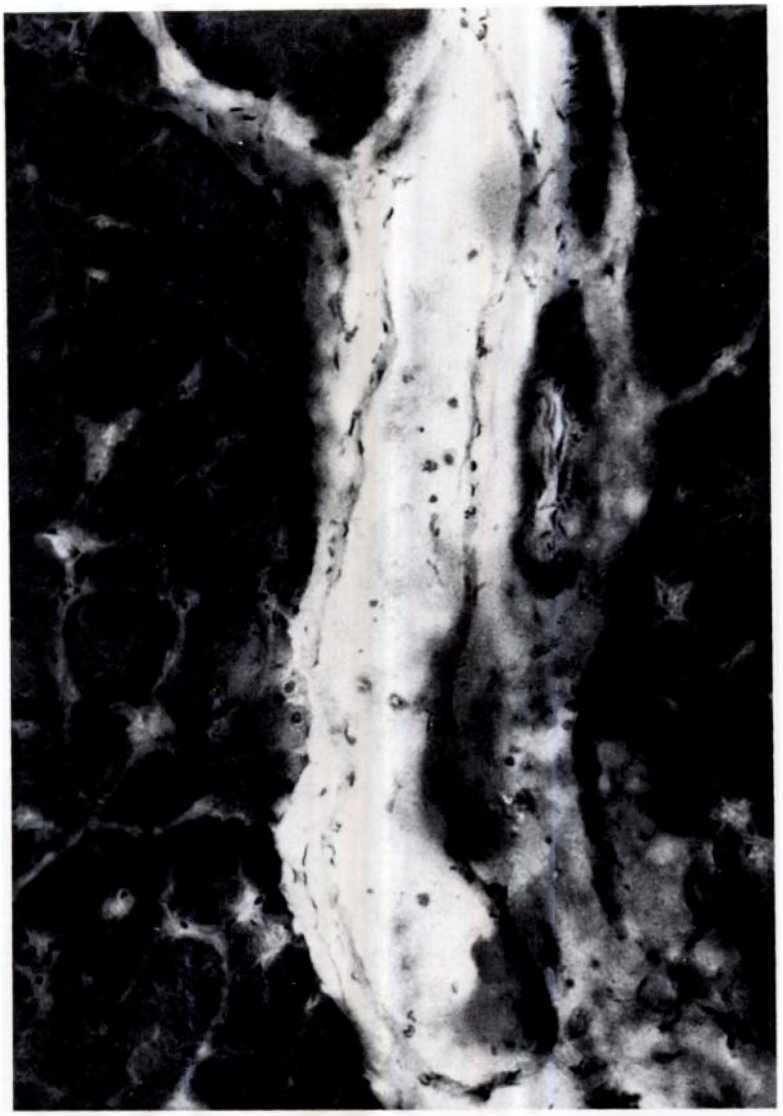


FIG. 9. Large thin walled vessel in section of rat submaxillary gland after pilocarpine stimulation and incubated on a 3% film for 20 min. Starch-PAS. ×250.

amylase. In the stimulated gland, there is histochemical evidence of a higher concentration of amylase in the blood vessels than in unstimulated gland. This evidence is that activity is demonstrable on 5% films after 10 min of incubation, whereas in the unstimulated gland no similar activity is seen after 30 min of incubation. Furthermore, on 3% films diffusion around the vessels is more pronounced in stimulated than in unstimulated glands treated under the same conditions.

These observations confirm the views of Junqueira *et al.* (2) and Sreebny and Meyer (9). With this technique, there is no histochemical evidence of synthesis of new enzyme in the stimulated glands as was suggested by Schneyer and Schneyer (5). We have considered the possibility that activity in the secretory acini or granular convoluted tubules may be masked by overstaining with PAS-positive material in these cells when the starch films are stained. This certainly may be the case with the sublin-

gual gland, the cells of which are intensely PAS-positive. This is unlikely, however, to be happening in the submaxillary gland where the cells contain no more PAS-positive material than do the cells of the parotid, and there is no problem about demonstrating amylase activity in the parotid acini. The possibility of a false negative result of this kind could possibly also be ruled out by staining the starch film with iodine instead of PAS. We find, however, that the iodine-staining procedure is generally unsatisfactory and tends to produce false positive results because it fades so rapidly.

We have also considered the possibility that the substrate film technique may be insufficiently sensitive to show very low levels of activity in the submaxillary secretory acini. This is an important consideration in view of the fact that in stimulated parotid large areas of undigested film are seen and yet quantitatively

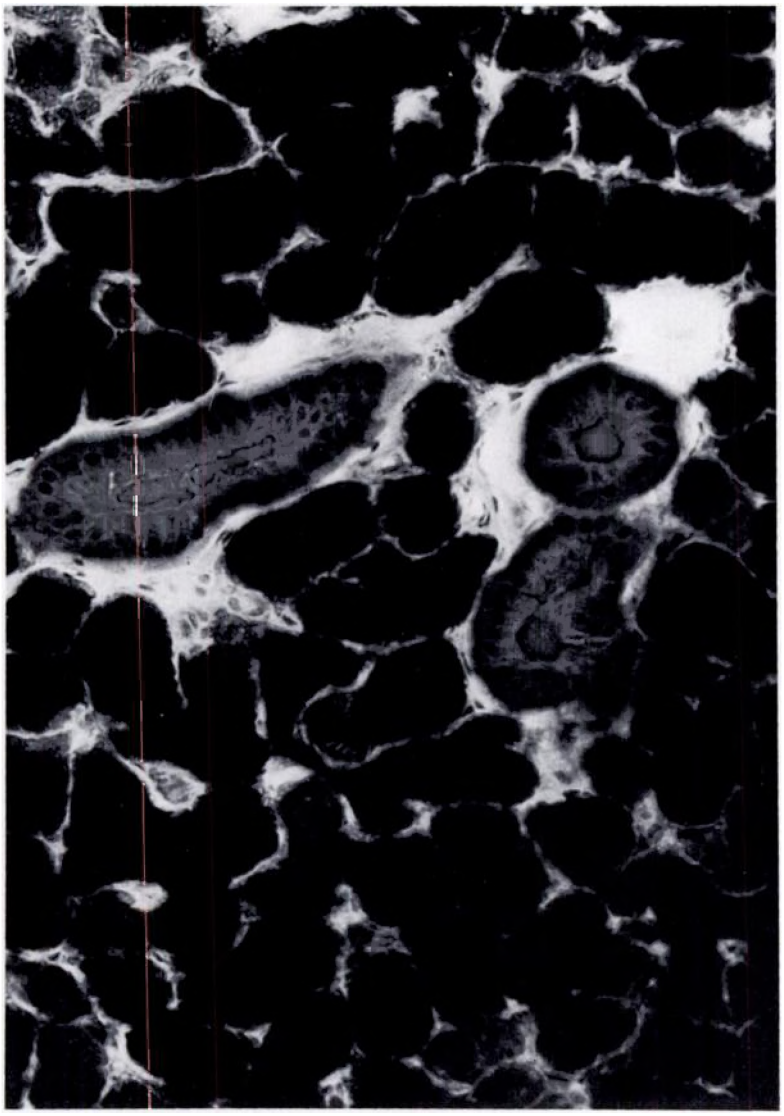


FIG. 10. Section of rat sublingual gland incubated on 3% starch film for 10 min. The PAS-positive secretory acini stain in addition to the starch film. Contents of blood vessels are positive for amylase; contents of duct lumina are negative. Starch-PAS. ×250.

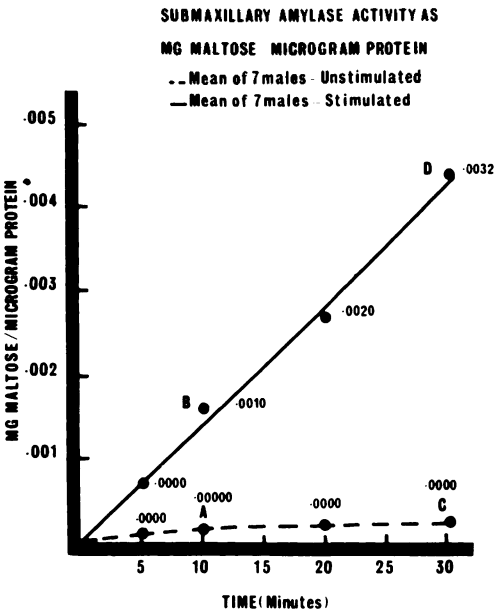


FIG. 11. Rat submaxillary amylase activity in fasting and pilocarpine-stimulated animals. The scale on the ordinate axis is $^1/_{100}$ that used to chart activity in the parotid. Unstimulated submaxillary shows negligible activity over an incubation period of 30 min. Activity in stimulated submaxillary increases linearly over this period. Mean amylase activity in unstimulated submaxillary glands of seven rats at 10 min is 0.00016 ± 0.00000 (S.D.) mg maltose/μg protein and 0.0016 ± 0.0010 in stimulated glands (points A and B, respectively). This difference is statistically significant ($t = 3.80$; $P < 1\%$). At 30 min the amylase activities are 0.00024 ± 0.00000 for unstimulated and 0.0044 ± 0.0032 for stimulated tissue (points C and D, respectively). This difference is statistically significant ($t = 3.44$; $P < 2\%$). Figures shown at points along the curves are standard deviations.

the level of amylase activity is still very many times greater than in stimulated submaxillary. We believe, however, that the explanation for this is that when pilocarpine stimulation leads to a 50% depletion of amylase in the parotid, the effect has not been produced evenly on all of the secretory units in the gland. Some groups appear, histochemically, to have been completely depleted, others only to a limited extent and still others not at all. The high levels of activity remaining in stimulated parotid are probably conferred by the undepleted acini. It would seem, moreover, that even if the activity present in the secretory acini of stimulated submaxillary is too low to be detected by the substrate film technique it must nevertheless be of a much lower order than that present in the blood.

The fact that we were consistently unable to demonstrate amylase activity in ducts (Figs. 6, 8 and 10) of stimulated glands is further evidence against a hypothesis that the acinar cells or granular tubules are synthesizing the enzyme. Junqueira *et al.* (2) showed that there was a negligible amount of amylase activity in the submaxillary saliva of carbamylcholine-stimulated rats.

The 10-fold increase in activity in sections of stimulated submaxillary after 10 min of incubation and the 18-fold increase after 30 min of incubation are of a similar order of magnitude shown by biochemical assay of whole gland. The range of readings found in the stimulated material in different animals may depend on the part of the gland from which the sections were cut. It was noticed that higher readings were obtained when the sections contained long segments of the large interlobular vessels.

There appears to be a decrease in total tissue protein in cryostat sections of salivary glands of pilocarpine-stimulated rats, particularly in the submaxillary. Whether or not this is a direct effect of pilocarpine on the mechanisms of protein synthesis and breakdown is not known.

Finally, it may be said that the experimental work of Chignell (1) does not appear to invalidate the hypothesis that the increase of amylase activity in the submaxillary-sublingual complex after pilocarpine stimulation results from an increase in the level of the enzyme in the blood.

In conclusion, these histochemical findings are consistent with the proposal of Junqueira *et al.* (2) that following cholinergic stimulation there are simultaneous increases in amylase activity of rat serum and glands and that this might be interpreted as a passage to the blood of enzymes produced by other glands under the same stimulus.

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