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Kallikrein, trypsin-like proteases and amylase in mammalian submaxillary glands

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Summary

1. The tissue concentration of kallikrein, trypsin-like proteases and amylase has been determined in the submaxillary glands of the cat, dog, rabbit, rat, hamster and guinea-pig.

2. Submaxillary gland extracts of the six species showed a wide variation in kallikrein content. The esterase activity in the extracts, however, did not correlate with the kininogenase activity of kallikrein. Both activities in each of the six species were affected by proteinase inhibitors but not in the same way.

3. Evidence is presented which indicates that trypsin-like proteases similar to those described in the rat, also occur in the hamster and cat submaxillary glands.

4. An even greater spread of amylase activity was observed between the species.

5. The assumption that mammalian submaxillary kallikreins are identical proteins is questioned.

Introduction

Despite many studies the physiological function of salivary kallikrein remains controversial (see Schachter, 1969). Since in exocrine glands macromolecules synthesized for secretion are mainly sequestered in intracellular organelles, in 1962 experiments were designed to examine whether kallikrein also was stored similarly in cells. Initial results clearly suggested that kalikrein was located intracellularly in specific membrane-bounded granules (Bhoola & Ogle, 1966). This finding was confirmed in subsequent studies in which the kallikrein-containing granules were characterized in considerably greater detail (Bhoola, 1968 ; Bhoola & Heap, 1970). Also, a similar subcellular distribution pattern was obtained for kallikrein in homogenates of the submaxillary glands of several mammalian species (Erdös, Tague & Miwa, 1968 ; Chang, Erdös, Miwa, Tague & Coalson, 1968 ; Bhoola, 1969).

Although it is now established that the serine protease kallikrein, like the digestive enzyme amylase, is stored within cells in specific organelles and secreted into saliva in response to physiological stimuli, the tissue content of submaxillary kallikrein has been determined only in a limited number of species. In addition, since trypsin-like enzymes have been isolated recently from the rat submaxillary gland (Riekkinen, Ekfors & Hopsu, 1966 ; Riekkinen, Ekfors, Hollmèn & Hopsu-Havu, 1967 ; Riekkinen, Ekfors & Hopsu-Havu, 1967), the question arises whether different kininogenases occur in the same mammalian submaxillary glands and what, if any, is their physiological role. The species distribution of serine proteases and other

secretory enzymes could relate to function and therefore in the present study the submaxillary gland content of kallikrein, trypsin-like proteases and amylase has been determined in the cat, dog, rabbit, rat, hamster and guinea-pig.

Methods

Both submaxillary glands from six cats and dogs, twelve rabbits, twenty-four rats and hamsters and forty guinea-pigs were removed under pentobarbitone anaesthesia. The glands from each species were washed several times in 0·9% NaCl to remove traces of blood, dried on filter paper, cut into small pieces, freeze-dried and pooled. The pooled, freeze-dried glands were extracted with 0·067 M Tris-HCl buffer, pH 8·0 (for measuring the trypsin-like proteases and kallikrein), Mg-free Tyrode solution (for the kininogenase activity of kallikrein) and Sörensen's buffer, pH 6·9 (for amylase). Triton X-100 (0·1 ml) was added to all the extracts except those used for determining the kininogenase activity of kallikrein. The extracts (50 mg freeze-dried tissue/ml) were centrifuged at 4,500 r.p.m. for 10 min and the supernatant used for enzyme measurements.

Measurement of enzymes and protein

Kallikrein

Kallikrein (EC 3.4.4.21) is defined as an endogenous enzyme (kininogenase) which rapidly and specifically liberates a kinin (kallidin) from kininogens (α2 globulins) in plasma. In this study both the kininogenase and the esterase activities of kallikrein were determined. The kininogenase activity was measured by the ability of kallikrein to release the nonapeptide, kallidin, from heated (61° C, 3 h) dialysed dog plasma which contained kininogen of suitable specificity and in adequate amount. In previous unpublished observations, dog plasma prepared in this manner was found to contain the highest kininogen content when compared with cat, human, rat, rabbit and guinea-pig plasma, the enzyme source being saliva and submaxillary gland extracts of the dog, cat, rabbit, rat and guinea-pig (see also Bhoola, 1961). In plasma heated in this manner both prekallikrein (precursor of kallikrein) and kininase (a peptidase that inactivates the kinins, kallidin and bradykinin, which are almost identical in structure and possess qualitatively similar biological activity) are destroyed. Furthermore, before assay, 1-cysteine (20 μg/ml) which is an inhibitor of tissue kininase was added to the extracts. In addition, before each test 1-cysteine (20 μg/ml), atropine (10 ng/ml) and mepyramine (10 ng/ml) were added. The substrate (0·15 ml, heated dialysed dog plasma) and the enzyme (0·1–0·4 ml, suitably diluted submaxillary gland extract) were added directly to the organ bath which had a constriction (1 mm internal diameter) at its base, in order to restrict the diffusion of added plasma and extract to a definite volume (see Schachter, 1956). The activity of the released kallidin was assayed against bradykinin (BRS, 640, Sandoz) on the isolated guinea-pig ileum. Subsequently extracts were dialysed against 0·9% NaCl at 4° C for 48 h and the kininogenase activity reassayed on the isolated guinea-pig ileum, rat duodenum, isolated uterus of stilboestrol primed rat (bath volume 12 ml) and the cat jejunum (bath volume 20 ml) (see Schachter, 1956; Ferreira & Vane, 1967). Each preparation was suspended in Mg-free Tyrode solution and gassed with 95% O_2–5% CO_2. Contractions were recorded on a smoked drum using a frontal writing lever. The kininogenase activity of the extract was expressed as ng synthetic bradykinin equivalents/mg tissue protein.

The esterase activity of kallikrein was measured on benzoyl arginine ethyl ester (BAEe) (see Trautschold & Werle, 1961; Trautschold, 1970). The assay medium consisted of 0·2 ml (6 mM) BAEe, 0·1 ml (30 mM) nicotinamide adenine dinucleotide and 0·02 ml (0·75 mg protein, 150 units) yeast alcohol dehydrogenase; 2·6–2·2 ml buffer solution (0·15 M semicarbazide hydrochloride, 0·15 M tetrasodium pyrophosphate and 0·044 M glycine, pH adjusted to 8·7 with 2 N NaOH) and 0·1–0·5 ml tissue extract were added to give a final volume in the cuvette of 3·02 ml. The reference cell (blank) contained 0·2 ml (6 mM) BAEe and 2·8 ml buffer solution. The increase in absorbance was followed at 25° C at 366 nm for 3 min in a Unicam S.P. 800 spectrophotometer. The rates of hydrolysis obtained with a partially purified preparation of human submaxillary kallikrein (95 KU/mg, kindly provided by Professor H. Moriya) and trypsin followed first order kinetics. In all cases the initial linear phase was used to calculate the rate of each reaction. An absorbance change of 0·0011 ΔE/min corresponds to 1 milliunit of BAEe hydrolysing activity which was expressed as milliunits/mg protein tissue extract. In order to assess the amount of BAEe hydrolysing activity arising from plasma trapped in the isolated gland, freshly collected plasma was tested; in the six species studied plasma contained less than 1 mU/mg protein of BAEe hydrolysing activity.

Trypsin-like proteases

Both kallikrein and trypsin are very active in hydrolysing synthetic esters of arginine. Submaxillary kallikreins unlike trypsin are believed, however, to have no action on synthetic amides except the biological plasma substrate. Highly purified porcine submaxillary kallikrein shows no activity on haemoglobin or nα-benzoyl (±)-arginine β-naphthylamide (Werle & Kaufmann-Boetsch, 1960). However, the kininogenase activity of submaxillary kallikrein (hypotensive action on the dog's blood pressure) appears to be paralleled by its BAEe splitting activity (Werle & Kaufmann-Boetsch, 1960). In order therefore to differentiate the kallikrein activity further and also to identify trypsin-like proteases in the submaxillary gland extracts the more highly specific trypsin substrate, benzoyl arginine *p*-nitroanilide (BAPA; Erlanger, Kokowsky & Cohen, 1961; see Trautschold, 1970), was used. Highly purified porcine (1,000 KU/mg, kindly provided by Professor E. Werle) and human ((240 KU/mg (2·08 μmol BAEe/min)/mg), kindly provided by Professor H. Moriya) submaxillary kallikreins were ineffective in hydrolysing BAPA.

In general, the assay medium consisted of 0·2 ml BAPA (0·01 M), 2·6 ml Tris-HCl buffer (0·2 M Tris-HCl and 0·025 M $CaCl_2$, pH 7·8) and 0·2 ml tissue extract. The rate of hydrolysis was followed at 405 nm at 25° C for 3 min on a Unicam S.P. 800 spectrophotometer. Changes in optical density were linearly related to the *p*-nitroaniline released in the incubation medium. The BAPA hydrolysing activity (0·0036 ΔE/min$\equiv$ImU) was calculated as milliunits/mg protein tissue extract.

Proteinase inhibitors

The kallikrein and trypsin-like activity of the tissue extracts was examined after incubation with aprotinin (Trasylol, Bayer) (5,000 KIU/mg or 10,000 KIU/ml, kindly provided by Professor E. Werle) and soybean (SBTI) (1 μg inhibits 1·6 ug trypsin, type 1–5), lima bean (1 μg inhibits 1·5 μg trypsin, type 11-L) and ovomucoid (1 μg inhibits 1 μg trypsin, type 11-0) trypsin inhibitors (Sigma). The inhibitor concentration used in these experiments blocked completely the enzymic activity of 200 μg

of trypsin. The BAPA hydrolysing enzymes were incubated with each of the four proteinase inhibitors. The incubation was carried out in the assay cuvette at room temperature for 20 minutes. The inhibitor concentration in the assay cuvette before the addition of the substrate was generally between 350–370 μg/ml for soybean, lima bean and ovomucoid trypsin inhibitors and 270–540 KIU/ml for aprotinin. The method of measurement of the BAEe and BAPA hydrolysing activity in the presence of inhibitor was identical to that already described. In the kininogenase experiments the extracts were preincubated with the various inhibitors in Tyrode solution. After 20 min aliquots of the incubation mixture were added to the organ bath and assayed. The inhibitor concentration varied between 100–2,500 μg/ml for the trypsin inhibitors and 500–2,000 KIU/ml for aprotinin. Furthermore, the peak anodal and cathodal enzyme activities on the paper electrophoretograms (see below) were also tested against the four inhibitors.

Amylase

Amylase activity was measured by the amyloclastic method using iodine (see Bhoola & Heap, 1970). A change in transmission from 0 to 50% in 5×10^3 min was defined as 1 milliunit of amylase activity which was calculated as milliunits/mg protein tissue extract.

Protein

This was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

Paper electrophoresis

The freeze-dried glands were extracted with 0·067 M Tris-HCl buffer, pH 8·0 (50 mg tissue/ml). The extract was centrifuged and the supernatant applied to Whatman No. 1 paper, 35 cm long and 5 cm wide. The electrophoresis was carried out in barbitone buffer pH 8·6 at 4° C for 10 h (6 mA ; 220 V). The papers were dried at room temperature. They were cut into 0·5 cm wide segments on either side of a 1 cm wide isopotential zone. The paper segments were eluted with 0·067 M Tris-HCl buffer pH 8·0. The eluates were tested for kininogenase, BAEe and BAPA activities. Fractions with peak activities were also tested after incubation with the proteinase inhibitors.

Results

Kallikrein

The kininogenase activity in submaxillary glands of the cat, dog, rabbit, rat, hamster and guinea-pig is shown in Table 1. Although the highest kinin-releasing activity was found in the rat, the guinea-pig and hamster contained comparatively high concentrations of kallikrein. No previous values have been reported for the rabbit, hamster and the guinea-pig, but the relative amounts in the cat, dog and rat are qualitatively similar to the original figures obtained by Werle and co-workers (see Frey, Kraut & Werle, 1950) who used the dog's blood pressure to assay kallikrein. Although the guinea-pig ileum is suitable for assaying kallikrein, interference from other analogous systems, for example renin-angiotensin, could not be ruled out. The presumed kininogenase activity was therefore further characterized on

the cat jejunum (see Ferreira & Vane, 1967) which shows a high degree of specificity for the released kallidin. Qualitative characterization of the kininogenase activity was further extended on assays on the rat uterus and the rat duodenum.

It is not known whether the kininogenase in submaxillary gland of the different mammalian species are identical proteins. Previous studies with aprotinin appear to indicate that there are differences between the mammalian submaxillary kallikreins. In this study further differences were observed with several proteinase inhibitors and these are shown in Table 2. The kininogenase activity in the rat was completely inhibited by aprotinin whereas that in the other species was relatively unaffected. Furthermore, about 70% of the kinin-releasing activity in extracts of cat, rabbit and rat submaxillary glands was blocked by soybean trypsin inhibitor. The inhibition of kinin releasing activity by SBTI was confirmed using the cat jejunum. None of the four proteinase inhibitors tested appeared to affect the kininogenase activity in the guinea-pig, hamster and the dog (see Table 2).

Typical records of the initial rate of hydrolysis of BAEe by extracts of rat and cat submaxillary glands are shown in Fig. 1 and the dose-response relationship obtained in each of the species is illustrated in Fig. 2. Although, like the kininogenase values, the esterase activity was highest in the rat and lowest in the rabbit (see Table 1), there was no quantitative correlation between the ratios of the two activities in each of the six species (see Table 3). The effect of proteinase inhibitors on the hydrolysis of BAEe by gland extracts is illustrated in Fig. 3 and the complete inhibition profile is shown in Table 4. The lack of correlation between the kininogenase and esterase activities seemed to suggest the presence of additional BAEe hydrolysing enzymes with no kinin-releasing activity.

TABLE 1. *Kallikrein content of submaxillary glands*

	Kininogenase		Esterase
	Guinea-pig ileum ((ngB/mgP)	Cat jejunum (ngB/mgP)	(mU/mgP)
Cat	3,099	1,346	146
Dog	258	194	39
Rabbit	135	75	21
Rat	32,025	20,815	11,090
Hamster	2,375	743	7,443
Guinea-pig	6,575	2,293	42

Enzyme activity expressed as ng synthetic bradykinin equivalents/mg protein tissue extract (ngB/mgP) and as milliunits/mg protein tissue extract (mU/mgP). Mean results of four-six experiments; each experiment represents the mean of multiple measurements performed on extracts prepared from pooled, freeze-dried submaxillary glands of each of the six species.

TABLE 2. *Action of inhibitors on the kininogenase activity of kallikrein*

	% Inhibition of kininogenase activity					
Proteinase inhibitors	Cat	Dog	Rabbit	Rat	Hamster	Guinea-pig
Aprotinin	0	0	34	100	0	0
Soybean	72	0	75	69	0	0
Lima bean	0	0	0	10	0	0
Ovomucoid	0	0	0	2	0	0

Mean results of three-five experiments.

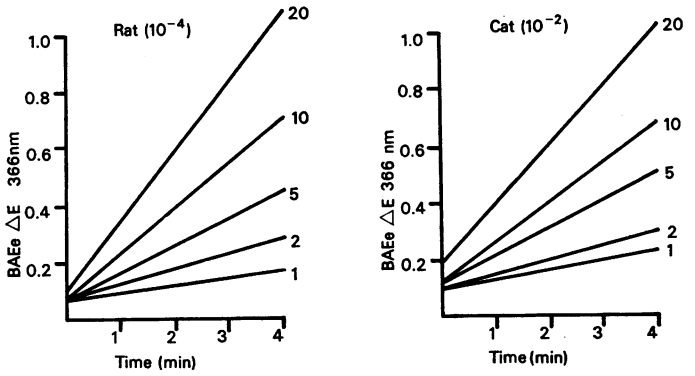

FIG. 1. Initial rates of hydrolysis of BAEe by Tris-HCl extract of rat and cat pooled, freeze-dried submaxillary glands. The figure associated with each rate corresponds to a dilution volume; the rat extract being equivalent to a 10,000-fold dilution (10^{-4}) and the cat extract to a 100-fold dilution (10^{-2}).

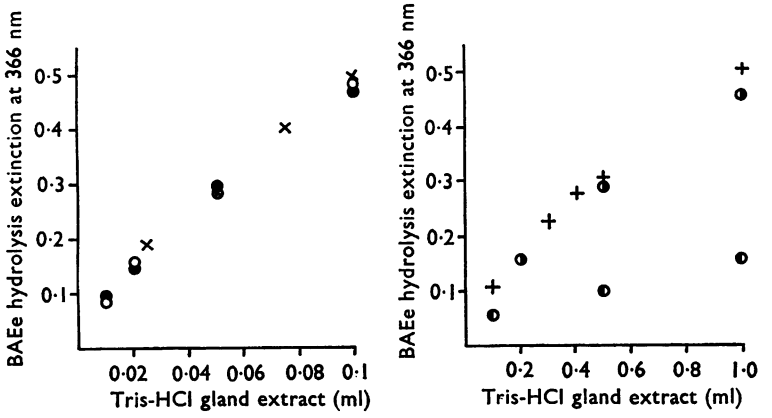

FIG. 2. Dose-response relationship between BAEe hydrolysis and the esterase activity of rat (○), hamster (×), cat (●), guinea-pig (+), dog (◑) and rabbit (◐) submaxillary gland extracts. Extracts of the rat were diluted 100-fold and hamster 50-fold before measurement.

TABLE 3. *Ratios of kininogenase and esterase activities*

Species	KGI/E	KCJ/E
Cat	21·2	9·2
Dog	6·6	4·9
Rabbit	6·4	3·5
Rat	2·8	1·8
Hamster	0·3	0·09
Guinea-pig	156·5	54·5

Kininogenase activity assayed on guinea-pig ileum KGI and cat jejunum KCJ. Esterase activity, E.

TABLE 4. *Effect of inhibitors on the esterase activity*

	% Inhibition of esterase activity					
Protease inhibitors	Cat	Dog	Rabbit	Rat	Hamster	Guinea-pig
Aprotinin	11	14	29	90	23	10
Soybean	71	8	43	30	0	0
Lima bean	0	0	0	10	0	0
Ovomucoid	0	0	0	7	5	0

Mean results of two-three experiments.

In preliminary experiments an attempt to separate these activities was unsuccessful. The electrophoretic profile of the esterase activity was similar and could not be differentiated from the kininogenase activity. The major migration of both activities, in particular the kininogenase activity, was towards the anode. The peak activities of the electrophoretograms when tested against proteinase inhibitors showed a qualitatively similar inhibition profile to that obtained with the gland extracts.

Trypsin-like proteases

Enzymes hydrolysing the synthetic substrate benzoyl arginine *p*-nitroanilide (BAPA) were detected in the submaxillary gland extracts of only the rat, hamster and the cat. The highest amounts were found in the rat and the least in the cat

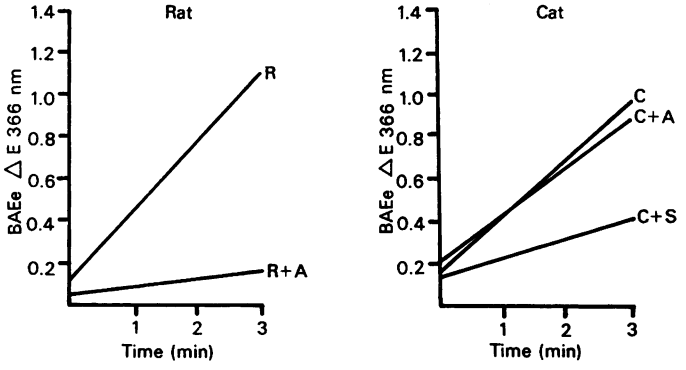

FIG. 3. Inhibition of the esterase (BAEe) activity in the Tris-HCl extract of rat submaxillary gland (R, control) by aprotinin (R+A) and of cat submaxillary gland (C, control) by aprotinin (C+A) and soybean trypsin inhibitor (C+S).

TABLE 5. *Concentration of secretory enzymes in submaxillary glands*

Species	Kallikrein* (ngB/mgP)	Trypsin-like proteases (mU/mgP)	Amylase (mU/mgP)	Protein (mg/g)
Cat	1,346	0·06	0	389
Dog	194	0	0	237
Rabbit	75	0	0·6	470
Rat	20,815	125	12·0	485
Hamster	743	0·8	0	480
Guinea-pig	2,293	0	1,436	371

Enzyme activity expressed as ng synthetic bradykinin equivalents/mg protein tissue extract (ngB/mgP, * measurement on cat jejunum) and as milliunits/mg protein tissue extract (mU/mgP). Mean results of three-six experiments; each experiment represents the mean of multiple measurements performed on extracts prepared from pooled, freeze-dried submaxillary glands of each of the six species. Zero activity indicates no hydrolysis of substrate by extracts after 20 min incubation in the case of trypsin-like proteases and after 120 min incubation in the case of amylase.

TABLE 6. *Action of inhibitors on trypsin-like proteases*

Proteinase inhibitors	% Inhibition of BAPA hydrolysis Rat	Hamster	Cat
Aprotinin	88	0	27
Soybean	87	0	18
Lima bean	30	0	6
Ovomucoid	0	0	0

Mean results of three experiments.

(Table 5). The affinity of the proteinase inhibitors for the BAPA hydrolysing enzymes in the three species is indicated in Table 6. Furthermore, the electrophoretic mobility of these enzymes differed slightly from that of the esterases but the differences were not of sufficient magnitude to conclude whether the same enzyme was responsible for the BAPA, BAEe and kininogenase activities.

Amylase

The submaxillary glands of the cat, dog and hamster contained no amylase. A small amount of activity was detected in the rat and even less in the rabbit, but by far the highest concentrations were observed in the guinea-pig (see Table 5). Unlike kallikrein and the trypsin-like proteases, amylase showed a dominant movement towards the cathode in the electrophoretograms of each of three species in which it was present.

Discussion

The kallikreins (EC 3.4.4.21) were discovered about 40 years ago (see Werle, 1970) and are now known to be serine proteases which belong to the same family of enzymes as trypsin (EC 3.4.4.4) and chymotrypsin (EC 3.4.4.5) (Habermann, 1962; Fiedler, Müller & Werle, 1969, 1970). Unlike the latter enzymes, the mammalian submaxillary kallikreins purified so far have a high degree of substrate specificity; they do not hydrolyse any naturally occurring proteins except the kininogens which are found in plasma and lymph. Furthermore, although purified hog and human salivary kallikreins are very effective in hydrolysing synthetic amino-acid esters (BAEe) they apparently show no activity on synthetic amides, in particular BAPA which is considered to be a more highly specific substrate for trypsin (see **Methods**). The presence, therefore, of BAPA hydrolysing enzymes in submaxillary gland extracts of the rat, hamster and the cat appear to indicate that either salivary kallikreins in these species are different from those in other mammalian species or that several different kininogenases (both kallikrein- and trypsin-like enzymes) occur in each of these species. In addition, it is possible that the extracts contain BAEe hydrolysing esterases with no kininogenase activity.

Recently three trypsin-like BAPA hydrolysing proteases (salivain, pH optimum 9·2–9·3; glandulain, pH optimum 8·0–8·2; and a third protease, pH optimum 6·9–7·1) and four kallikrein isoenzymes have been isolated and purified from rat submaxillary gland. Unfortunately the detailed biochemical studies have not been matched by adequate pharmacological characterization of these enzymes. From the published data it seems that salivain and the kallikrein-like BAPA splitting peptidases described by Riekkinen and his colleagues may have kininogenase activity (see Ekfors, Riekkinen, Malmiharju & Hopsu-Havu, 1967; Ekfors, Malmiharju, Riekkinen & Hopsu-Havu, 1967; Ekfors, Hopsu-Havu & Malmiharju, 1969).

The experiments described here on the effect of inhibitors on kininogenase activity also appear to indicate that different kallikreins occur in submaxillary glands. This study suggests that the kininogenase in the cat may be similar to that in the rabbit but different from that in the dog, guinea-pig and hamster. The possibility that the submaxillary kallikreins of various mammalian species are not identical proteins therefore clearly emerges from this study. But, suitable comparisons between the submaxillary kininogenases must await isolation, purification and complete chemical and pharmacological characterization of their properties in each of the species.

The close association of trypsin-like proteases with kallikrein in the submaxillary of the rat, hamster and the cat may be of functional importance. Furthermore, no adequate explanation has been advanced for the many fold differences in the concentration of kallikrein between the six species examined. Also of interest is that the highest kininogenase activity occurs in the rat submaxillary gland, in which the trypsin-like glandulain shows a remarkable dependence on androgens (Riekkinen & Niemi, 1968). These findings need to be considered in assessing the physiological role of kallikrein in submaxillary glands.

Histochemically, the cat and dog submaxillary glands are designated as mucous, the rabbit, hamster and rat as seromucous and the guinea-pig as serous (Shackleford & Klapper, 1962). In this study kallikrein was present in all of these species with the highest concentration in the rat and relatively high amounts in the guinea-pig, hamster and cat; in contrast amylase was detected mainly in the guinea-pig with remarkably little in the rat and rabbit and none in the cat, dog and hamster. The postulate of Shackleford & Wilborn (1968) that secretory enzymes are located in serous acini only therefore requires modification. Our study clearly shows that there is no correlation between the occurrence of the secretory enzymes kallikrein, trypsin-like proteases and amylase and the histochemical reactivity of the acinar cells for mucosubstances (see Dorey & Bhoola, 1971a, b).

It is now established that the secretory enzymes kallikrein (Bhoola & Heap, 1970) and amylase (Schramm & Danon, 1961; Schramm, 1967) are stored intracellularly in membrane-bound organelles. In addition, the trypsin-like proteases may also be sequestered in similar granules. After appropriate stimuli the granules containing the secretory enzymes fuse with the luminal membrane of the acinar cell and empty their contents into the ducts. Under physiological conditions kallikrein, like amylase and perhaps the trypsin-like proteases, is secreted into saliva and its primary function may reside in the submaxillary gland duct or the alimentary tract.

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