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KALLIKREIN IN GRANULES OF THE SUBMAXILLARY GLAND*

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Abstract—A substantial amount of the kallikrein present in the submaxillary gland of the rat was found in granules. The granules were separated in discontinous density gradient centrifugation. Kallikrein was assayed and identified by measuring its esterase activity in the presence and absence of various inhibitors and by using tests of bioassays *in vitro* and *in vivo*. The granules lysed easily; they were more stable at room temperature than in the cold. Phase contrast and mainly electron microscopic investigations revealed that they resemble the zymogen granules. A fraction of the available kallikrein in the gland was firmly bound to particles and sedimented in the ultracentrifuge at high speed.

THE ROLE of kallikrein in regulating the blood flow in salivary glands has been debated for many years.[1–4] Studying the subcellular distribution of kallikrein in these tissues may be of interest in establishing the physiological importance of the presence of the enzyme. Of all tissues, the submaxillary gland of the rat has the highest kallikrein content.[5] This communication describes the presence of kallikrein in granules of the submaxillary gland of the rat.

MATERIALS AND METHODS

The activity of kallikrein was determined by chemical and biological assay methods. The hydrolysis of 5×10^{-4} M benzoyl-L-arginine ethyl ester (BAEe) by the enzyme was followed at room temperature in a Cary UV recording spectrophotometer at $\lambda = 2530$ Å.[6] The pH of the reaction was 8·0 in a 0·05 M triethanolamine buffer. The hydrolysis of 1×10^{-3} M benzoyl-DL-arginine *p*-nitro-anilide (BAPA) was measured at $\lambda = 4050$ Å in 0·1 M Tris buffer, pH 7·9.[7] In routine assays the BAEe esterase activity was registered after 0·1% Triton X-100 was added to the homogenate and the latter was diluted 1 : 1500 or 1 : 15,000 (v/v).

The biological activity of kallikrein *in vitro* was established by assaying on the isolated rat uterus the amount of kinin released by the enzyme. The substrate was human plasma heated for 2 hr at 56°. The kallikrein preparation contained 1,10-phenanthroline[4] to inhibit the enzymatic inactivation of the kinin by kininases present. The incubation mixture contained: 0·1 ml enzyme; 0·1 ml of 1,10-phenanthroline, 1×10^{-2} M; 0·3 ml of 0·2 M Tris, pH 7·4; and 0·5 ml of heated plasma. The sources of kallikrein, fractions of the submaxillary gland, were diluted 1:500 to 1:4000 (v/v) in the system.

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Samples were withdrawn for the assay every 10 min. Because rat kallikrein contracts rat uterus *in vitro*, 1000 units of Trasylol were added to the incubation mixture prior to injecting it into the muscle bath. Trasylol blocks the oxytocic activity of rat kallikrein. The hypotensive effect of i.v. injections of kallikrein was registered in the femoral artery of the dog by means of a Statham pressure transducer connected to a Grass polygraph. In control studies, a commercial kallikrein preparation (Padutin) containing 10 units per ampule was used.

Amylase was determined according to Searcy *et al.*[8] The substrate was soluble starch. Protein content was measured according to Lowry *et al.*[9]

Kallikrein was extracted from 1·6 g of homogenized submaxillary glands by stirring it in 16 ml of distilled water at 1° for 30 min. The pH was adjusted to 4·5 with HCl and the stirring continued for another hour. The precipitate was removed in the centrifuge at 5000 *g* in 20 min. The supernatant was stirred for 2 hr with 5 g DEAE-cellulose (Whatman) at pH 7. The DEAE-cellulose was washed on a sintered glass funnel with 100 ml water and the active fraction was eluted with five 10-ml portions of 0·4 M phosphate buffer, pH 6·0. This procedure is based on the method used by Moriya *et al.*[10] for purifying pancreatic kallikrein.

When initial experiments suggested a higher enzymatic activity and the presence of more granules in the gland of male rats than in the female, male rats older than 120 days were sacrificed. The fresh minced glands were homogenized in a Dounce hand homogenizer in 0·25 M sucrose buffered with 0·005 M Tris, pH 7·4. The tissues were placed in an ice bath and a 10% (w/v) homogenate was prepared. The homogenate was filtered through four layers of gauze. The unbroken tissues and cells were removed in a Sorvall RC-2 refrigerated centrifuge at 120 *g* for 5 min. The step was repeated once. The supernatant was centrifuged at 480 *g* for 20 min. The precipitate was washed with sucrose solution and resedimented at 480 *g*. The washed precipitate obtained at 480 *g* contained granules. This granular preparation is called Extract I.

The supernatant was centrifuged again at 10,800 *g* for 1 hr and at 144,000 *g* for 1 hr. The latter operation was repeated after washing the sediment. A Spinco L-2 65 ultracentrifuge was used in this procedure.

Extract I was further fractionated by differential centrifugation in a discontinuous density gradient according to Siekevitz and Palade.[11] An International refrigerated centrifuge with swinging bucket head was used for 4 hr at 2000 *g*. Ten ml Extract I in 0·25 M sucrose was layered on top of sucrose solutions in the centrifuge tube. The molarity and volume of the sucrose solutions from top to bottom were: 1·02 M, 4·7 ml; 1·60 M, 4·7 ml; 1·84 M, 3·3 ml. The granules were found on the boundary between the 1·60 M and 1·84 M solutions. This active granular fraction is called Extract II.

The granules were investigated by phase contrast microscopy, light microscopy and electron microscopy. Phase contrast microscopy of the extracts was done in a Leitz instrument at 500X magnification. For light microscopy, sections of 1–2 μ were stained with PAS (periodic acid Schiff's reaction)[12] and photographed with a Zeiss photomicroscope.

For electron microscopy the granules in Extract II were fixed for 15 min with glutaraldehyde buffered at pH 7·4 with 0·1 M sodium cacodylate. The fixed granules were sedimented in the centrifuge and washed with 0·2 M sucrose buffered as indicated above. The preparation was postfixed in Zetterquist's fixative for 45 min; it was

subsequently dehydrated in a graded series of ethanol and embedded in Epon 812. The blocks were polymerized for 48 hr at 60°. The specimens were sectioned with a Porter-Blum ultramicrotome and stained with lead citrate[13] and uranyl acetate.[14] The sections were examined with an RCA EMU-3F electron microscope. The appropriate fields were photographed.

Three times recrystallized trypsin was purchased from Worthington Biochemical Corp. and trypsin inhibitors from Nutritional Biochemical Corp. Trasylol was donated by FBA Medical Research.

RESULTS

Separation of granules. Table 1 shows the distribution of esterase activity in the homogenized gland. BAEe was hydrolyzed at a rate of 5400 μmole/min/*g* tissue. About 8 per cent of the total activity was discarded with the 120 *g* sediment that contained some unbroken cells and tissues. Roughly one-fourth of this esterase was recovered in the washed 480 *g* sediment, in Extract I. Sixty-eight per cent of esterase was in the soluble phase after the 480 *g* centrifugation. Of this 2 per cent sedimented at 10,800 *g* and less than 0·1 per cent was found in the 144,000 *g* washed sediment.

TABLE 1. DISTRIBUTION OF BAEe ESTERASE ACTIVITY AMONG FRACTIONS OF THE HOMOGENIZED SUBMAXILLARY GLAND*

Source of enzyme	Hydrolysis of BAEe	
	(% of total activity)	(μmole/min/*g* tissue)
Crude homogenate	100	5400
Sediment, 120 *g*	8	
Sediment, 480 *g*	24	
Sediment, 10,800 *g*	2	
Sediment, 144,000 *g*	<0·1	
Final supernatant	66	

* Sediments were washed once with sucrose solution.

The granules in Extract I were unstable and lysed on repeated washing. Since it was shown that zymogen granules of the parotid gland containing amylase[15] were more stable at room temperature than at 1°, Extract I was divided into two portions; one part was stored at room temperature in 0·25 M sucrose for 15 min, the other at 1°. After 15 min, 85 per cent of the activity could be resedimented at room temperature while only 12 per cent of enzyme was recovered in the sediment from the sample standing in an ice bath. Triton X-100 or suspension of the granules in hypotonic solution released the bound enzyme in both samples (Table 2).

Extract II, which was obtained after discontinuous density gradient centrifugation, contained about 48 per cent of the esterase activity of the total applied in this procedure. About 28 per cent was found in the top layer in the centrifuge tube (0·25 M sucrose) and 11 per cent in the bottom layer. The other layers combined were responsible for only 13 per cent of the enzymatic action.

The granules in Extract II were stable only in hypertonic sucrose solution. They disappeared when the molarity was lowered to 0·25 M.

TABLE 2. STABILITY OF GRANULES AT ROOM TEMPERATURE AND IN THE COLD*

Procedure	Per cent of BAEe esterase released from granules	
	At 1°	At 25°
Standing for 15 min	85	12
Standing + Triton X-100	—	98
Standing + H_2O	—	94

* The granules were obtained from one pair of glands (average weight 0·7 g) homogenized and centrifuged at room temperature.

Microscopic investigation of the granules. The granules were studied by phase contrast, light and electron microscopy. Extract I contained vesicles that could be seen under a phase contrast microscope at 500X magnification. The dissolution of these bodies was also observed when a drop of Triton X-100 was added to the slide.

Photomicrographs of Extract I revealed that it consists of PAS-positive material. This is taken as an indication that the extract contains carbohydrate. Secretory granules are known to be PAS-positive.[16]

The granules were further concentrated in discontinuous density gradient centrifugation (see Methods). Extract II contained a large number of granules ranging up to 2 μ in diameter. Electron microscopy revealed that the granules vary in size and look similar to zymogen granules of the pancreas[17] (Fig. 1).

Identification of kallikrein. Because, in addition to kallikrein, trypsin[6] and other enzymes of the submaxillary gland[18, 19] hydrolyze BAEe, kallikrein in the granules had to be differentiated from other esterases. This was done by using various trypsin inhibitors and employing bioassay for the identification of kallikrein. As shown in Tables 3, 4 and 5, the major portion of BAEe esterase action in the granules in Extracts I and II can be attributed to kallikrein.

The hydrolysis of BAEe in Extracts I and II was inhibited 95 and 100 per cent by the kallikrein and proteolytic inhibitor Trasylol, but only 16 and 5 per cent by soybean trypsin inhibitor (SBTI). Increasing the concentration of SBTI from 67 to 134 μg/ml did not increase the inhibition. Other trypsin inhibitors were even less effective against Extract I than SBTI. In control studies, 67 μg/ml SBTI inhibited trypsin (1·7 μg/ml)

TABLE 3. PER CENT INHIBITION OF BAEe ESTERASE ACTIVITY*

Source of enzyme	Rate of hydrolysis of BAEe (μmole/min/mg protein)	Inhibitor*				
		Soybean trypsin inhibitor (67 μg/ml)	Trasylol (100 units/ml)	Ovomucoid trypsin inhibitor (67 μg/ml)	Lima bean trypsin inhibitor (67 μg/ml)	DFP (5×10^{-3} M)
Extract I	163	16	95	15	4	96
Extract II	173	5	100	—	—	—
Trypsin	35	98	—	—	—	—

* Uninhibited enzyme = 100%.
† The inhibitors were preincubated with the enzyme for 30 min.

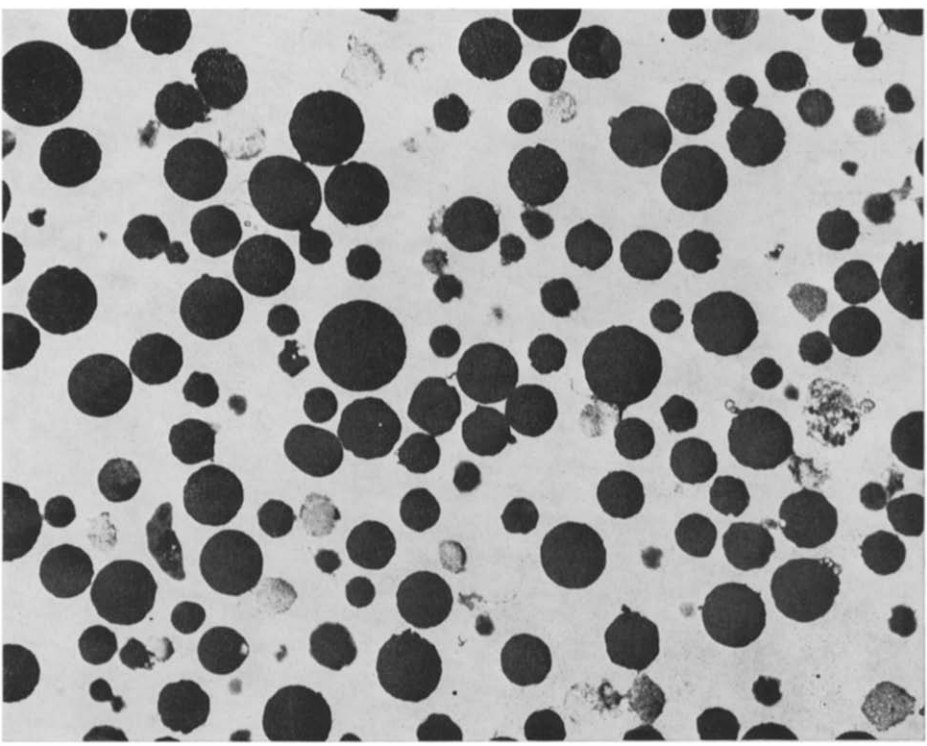

FIG. 1. Electron photomicrography of granules from submaxillary gland of rat that contain kallikein 5,250X magnification). Granules were separated in discontinous density gradient centrifugation.

completely with BAEe substrate. As expected, DFP stopped the enzymatic hydrolysis of BAEe (Table 3).

The concentration of Trasylol used was 100 units/ml; this is the equivalent of approximately 17 μg/ml of pure inhibitor.[20]

The esterase of Extract I had a pH optimum at 9. The activity dropped fairly steeply below neutrality (Fig. 2).

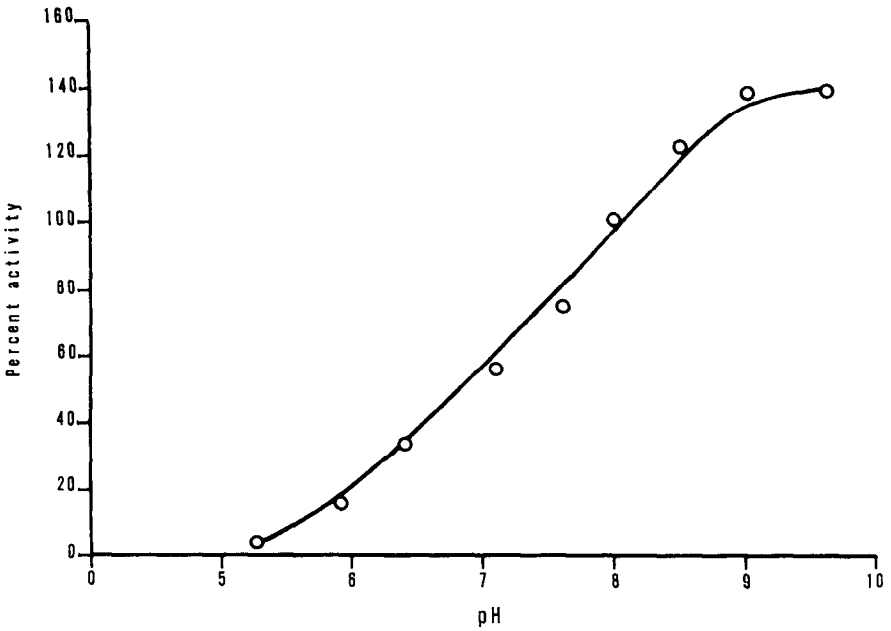

FIG. 2. pH curve of submaxillary kallikrein in Extract I. The following buffers were used: pH 5·5, 0·1 M acetate; pH 5·9 and 6·4, 0·2 M phosphate; pH 7·1, 7·6 and 8·5, 0·2 M Tris; pH 8, 0·05 M triethanolamine; pH 9 and 9·6, 0·05 M glycine.

The esterase of the crude homogenate was concentrated by batchwise elution of the adsorbed enzyme from DEAE-cellulose. About 70 per cent of the activity of the first homogenate was recovered from the cellulose. The activity of the enzyme increased about 13-fold in the 0·4 M phosphate buffer eluate over the crude when the rate of hydrolysis of BAEe by the 2780 Å absorbing material was compared. This concentrated kallikrein was also tested in bioassays *in vitro* and *in vivo*, together with the other sources of the enzyme.

BAPA was hydrolyzed much more slowly by Extract I than BAEe. The rate of hydrolysis of BAPA was 0·144 μmole/min/mg protein, that is about 0·1 per cent of the rate of cleavage of BAEe by the same enzyme (Table 3). SBTI inhibited (34 per cent) the splitting of BAPA more than the esterase action of the enzyme (Table 3).

Amylase. Extract I contained very little amylase. One g of crude homogenized gland released 0·7 mg of reducing sugar from the starch at 37° in 1 min. Only 2–3 per cent activity was recovered in the granular fraction; the rest was in the supernatant.

Bioassay. Further identification of kallikrein was done in biological experiments which are summarized in Tables 4 and 5.

Extracts I and II, kallikrein concentrated by means of adsorption on DEAE-cellulose and a kallikrein obtained at high speed (144,000 *g*) centrifugation in the

washed precipitate (microsomal kallikrein), all released a kinin from heated human plasma. In Table 4 the kinin assayed is expressed in bradykinin equivalents. The values were interpolated to 1 hr from the initial steady rates. As with BAEe substrate, Trasylol inhibited the enzyme, while SBTI was relatively inactive. The uterine activity of the peptide released rapidly disappeared after purified pancreatic carboxypeptidase B[4] was added to the solution.

TABLE 4. RELEASE OF KININ FROM HUMAN PLASMA BY KALLIKREIN OF THE SUBMAXILLARY GLAND

Source of kallikrein	Concentration (μg protein/ml)	μg of bradykinin* equivalent released in 1 hr from 0·5 ml plasma	Per cent inhibition†	
			SBTI‡ (100 μg/ml)	Trasylol (100 units/ml)
Extract I	1·0	1·5 (0·9–2·1)	15	77
Extract II	0·6	1·3 (0·9–1·6)	11	87
Concentrated kallikrein	0·8	1·9 (1–3·5)	9	76
Microsomal fraction (144,000 *g* sediment)	19	1·0 (0·6–1·2)	12	78

* Calculated from the initial steady rate. The figures in parentheses show the range.
† The inhibitors were preincubated with the enzyme for 30 min.
‡ Soybean trypsin inhibitor.

TABLE 5. LOWERING OF THE SYSTEMATIC ARTERIAL BLOOD PRESSURE OF DOG BY KALLIKREIN OF THE SUBMAXILLARY GLAND

Source of kallikrein	FU/mg protein*	Per cent inhibition	
		SBTI† (100 μg/ml)	Trasylol (500 units/ml)
Extract I	48 (30–78)	28	93
Extract II	78 (64–103)	27	95
Concentrated kallikrein	118 (101–134)	27	94

* FU = Frey units; the figures in parentheses show the range.
† Soybean trypsin inhibitor.

The Extracts I and II and the concentrated submaxillary kallikrein lowered the systemic arterial blood pressure of anesthesized dog. Extracts I and II contained 48 and 78 FU (Frey units) kallikrein/mg protein. The hypotensive action of the extracts was inhibited only 27 and 28 per cent by 100 μg/ml SBTI, but was almost completely inhibited by Trasylol.

We saw in exploratory studies that rat submaxillary kallikrein is hypotensive in other animals such as the rabbit.

DISCUSSION

These experiments indicate that a major portion of the particle-bound BAEe esterase activity in the submaxillary gland can be attributed to kallikrein. The subcellular particles were identified as granules of varying size and looked similar to zymogen granules.[17] The possibility of a nonspecific adsorption of kallikrein by the particles can be excluded.

Similarly to kallikreins from other glandular tissues,[4] the rat submaxillary kallikrein is inhibited by Trasylol, but not significantly by SBTI. The enzyme hydrolyzes BAEe 1000 times faster than BAPA. The optimum pH for kallikrein is 9. The enzyme is very active in releasing kinin from plasma kininogen and in lowering the blood pressure of experimental animals. It can be purified by the same techniques as pancreatic kallikrein.[10] The active fraction of the homogenate in Extract I or II was more hypotensive than trypsin[4, 21] and hydrolyzed BAEe faster[6, 22] when calculated on the basis of protein content. The high kallikrein content of the granular fractions, 48 and 78 FU/mg protein (Table 5), can be even more appreciated if we consider that highly purified hog submaxillary kallikrein preparations contained 200–510 FU/mg.[23] The rat glandular kallikrein is oxytocic on the isolated rat uterus.

About one-fourth of the total esterase activity of the homogenized gland was found in the granules isolated at 1° in isotonic sucrose. Since these granules are very sensitive and break easily, the intact gland probably contains a much higher percentage of kallikrein within the vesicles. It was shown that the granules are much more stable at room temperature than in the cold.

A small fraction of the total kallikrein was in the washed precipitate that sedimented at 144,000 *g* like the microsomal fraction. Possibly this kallikrein was still bound to broken membrane particles.

Kallikrein may occur in subcellular particles in other glands and other animals, as suggested by Bhoola and Ogle.[24] These authors fractionated the submaxillary gland of the guinea pig. Kallikrein activity was found in a low speed sediment in the nuclear fraction. Although their experimental conditions did not allow them to distinguish between "free" and "bound" kallikrein in the nuclear fraction, the authors assumed that the kallikrein was held in vesicles similar to zymogen granules.

The gland we studied had very little amylase activity.[25] It is known that, for example, the parotid gland of the rat has about 10,000 times more amylase than the submaxillary gland.[26] Almost none of the enzyme sedimented with kallikrein.

It was shown by Werle and Roden[27] in 1936 that the submaxillary gland of various animals contains more kallikrein than other salivary glands. Several other enzymes that can hydrolyze BAEe were found in the submandibular gland.[18, 19, 22] Although one of them (salivain) is hypotensive,[28] it was claimed to be different from kallikrein.

The role of the kallikrein–kinin system in higher animals is still a puzzle. It was suggested that in the salivary and other glands nervous stimulation leads to the release of kallikrein and by this mechanism causes vasodilation.[2, 4] Other investigators, however, disagree.[1, 3] Our present publication proved that a substantial amount of kallikrein is in granules in the submaxillary gland. It would be of interest to probe the role of kallikrein in functional vasodilation after the gland is degranulated.

Others found the sympathetic neurotransmitter originating from nerve endings to be in granules.[29] These granules that contain norepinephrine are different from the kallikrein granules. It is known that sympathetic stimulation greatly increases the kallikrein content of the saliva.[30] Possibly, when the sympathetic neurotransmitter is released from its storage granules, it acts on another type of intracellular particles to liberate kallikrein. This speculation, however, would require experimental confirmation.

The presence of such large amounts of kallikrein in the gland studied suggests another more remote function for the enzyme. Kallikrein-like enzymes are found in venoms originating from snake venom glands.[31] The kinins released by kallikreins cause

hypotension, pain and increase in capillary permeability.[4] It might be assumed that the kallikrein present in the saliva and in the submaxillary gland of rat can have a residual function somewhat similar to snake venom and contributes to the defense of the animal. It may be fortuitous, but another biologically active protein, a nerve growth factor, was found in snake venom first and in the submaxillary gland of rodents later.[32]

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REFERENCES

1. M. SCHACHTER, in *Hypotensive Peptides* (Eds. E. G. ERDÖS, N. BACK and F. SICUTERI), p. 275. Springer-Verlag, New York (1966).
2. S. M. HILTON, in *Hypotensive Peptides* (Eds. E. G. ERDÖS, N. BACK and F. SICUTERI), p. 281. Springer-Verlag, New York (1966).
3. M. E. WEBSTER, in *Hypotensive Peptides* (Eds. E. G. ERDÖS, N. BACK and F. SICUTERI), p. 263, Springer-Verlag, New York (1966).
4. E. G. ERDÖS, *Adv. Pharmac.* **4**, 1 (1966).
5. E. WERLE, in *Polypeptides Which Affect Smooth Muscles and Blood Vessels* (Ed. M. SCHACHTER), p. 199. Pergamon Press, Oxford (1960).
6. H-U. BERGMEYER, Ed., *Methods of Enzymatic Analysis*, p. 815. Academic Press, New York (1963).
7. W. NAGEL, F. WILLIG, W. PESCHKE and F. H. SCHMIDT, *Hoppe-Seyler's Z. physiol. Chem.* **340**, 1 (1965).
8. R. L. SEARCY, S. HAYASHI and J. E. BERK, *Am. J. clin. Path.* **46**, 582 (1966).
9. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
10. H. MORIYA, K. YAMAZAKI, H. FUKUSHIMA and C. MORIWAKI, *J. Biochem.* **58**, 208 (1965).
11. P. SIEKEVITZ and G. E. PALADE, *J. biophys. biochem. Cytol.* **4**, 203 (1958).
12. B. P. LANE and D. L. EUROPA, *J. Histochem. Cytochem.* **13**, 579 (1965).
13. E. S. REYNOLDS, *J. cell Biol.* **17**, 208 (1963).
14. M. L. WATSON, *J. biophys. biochem. Cytol.* **4**, 475 (1958).
15. M. SCHRAMM, B. EISENKRAFT and E. BARKAI, *Biochim. biophys. Acta* **135**, 44 (1967).
16 A. S. V. BURGEN and N. G. EMMELIN, *Physiology of the Salivary Glands*. Arnold, London (1961).
17. L. J. GREENE, C. H. W. HIRS and G. E. PALADE, *J. biol. Chem.* **238**, 2054 (1963).
18 P. J. RIEKKINEN, T. O. EKFORS and V. K. HOPSU, *Biochim. biophys. Acta* **118**, 604 (1966).
19 P. J. RIEKKINEN, T. O. EKFORS and V. K. HOPSU-HAVU, *Enzymologia* **32**, 110 (1967).
20. R. VOGEL, I. TRAUTSCHOLD and E. WERLE, *Natürliche Proteinasen-Inhibitoren*. p. 59. Thieme, Stuttgart (1966).
21. E. WERLE, M. M. FORELL and L. MAIER, *Arch. exp. Path. Pharmak.* **225**, 369 (1955).
22. R. A. ANGELETTI, P. U. ANGELETTI and P. CALISSANO. *Biochim. biophys. Acta* **139**, 372 (1967)
23. E. WERLE and I. TRAUTSCHOLD, *Ann. N.Y. Acad. Sci.* **104**, 117 (1963).
24. K. D. BHOOLA and C. W. OGLE, *J. Physiol. Lond.* **184**, 663 (1966).
25. L. H. SCHNEYER and C. A. SCHNEYER, *Ann. N.Y. Acad. Sci.* **85**, 189 (1960).
26. L. M. SREEBNEY and J. MEYER, Eds., *Salivary Glands and Their Secretions*, p. 103. Pergamon Press, New York (1964).
27. E. WERLE and P. v. RODEN, *Biochem. Z.* **286**, 213 (1936).
28. T. O. EKFORS, T. MALMIHARJU, P. J. RIEKKINEN and V. K. HOPSU-HAVU, *Biochem. Pharmac.* **16**, 1634 (1967).
29. U. S. v. EULER, S. ROSSELL and B. UVNÄS, (Eds.) *Mechanisms of Release of Biogenic Amines*, Pergamon Press, Oxford (1966).
30. C. MORIWAKI, S. BEILENSON and M. SCHACHTER, *Nature, Lond.* **217**, 271 (1968).
31. T. SUZUKI, S. IWANAGA, S. NAGASAWA and T. SATO, in *Hypotensive Peptides* (Eds. E. G. ERDÖS, N. BACK and F. SICUTERI), p. 149. Springer-Verlag, New York (1966).
32. S. COHEN, *Proc. natn. Acad. Sci. U.S.A.* **46**, 302 (1960).