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Bradykinin
cystic fibrosis of the pancreas
enzymic activity
kallikrein
kininase
saliva

Increased Kallikrein Content of Saliva from Patients with Cystic Fibrosis of the Pancreas

A Theory for the Pathogenesis of Abnormal Secretions

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Extract

An investigation of the bradykinin system in cystic fibrosis of the pancreas was undertaken because of the potential role of bradykinin in the function of glandular tissues and in the mediation of electrolyte transport. Patients with cystic fibrosis of the pancreas (CFP) and control subjects (CS) were studied for evidence of excessive formation or impaired inactivation of bradykinin in plasma, saliva and urine. Bradykinin, kininase and kallikrein activities were assayed by means of a modified Schultz-Dale apparatus utilizing the uterus of rats in estrus.

The bradykinin assay detected as little as 0.01 μg bradykinin (4×10^{-4} μg/ml bath solution). The height of uterine contraction was proportional to the logarithmic concentration of bradykinin with maximum contraction occurring with 1–10 μg bradykinin.

Free bradykininlike activity in urine, saliva or plasma specimens from 11 patients was the same as that from 21 normal controls. In saliva specimens from CFP or CS no bradykininlike activity was detected, whereas in urine there was slight activity in approximately 60% of the samples. Fresh heparinized plasma of both patients and control subjects developed spontaneous bradykinin activity when added to the muscle bath; this type of activity could be prevented by shaking the plasma with glass beads and incubating at 37° for 30 min. All of the 'glass-shaken' plasma specimens developed normal-appearing bradykinin activity when activated by salivary kallikrein. The presence of bradykininlike activity in urine was unrelated to proteinuria since none of the patients with activity in their urine had detectable proteinuria. Five other patients with the nephrotic syndrome and heavy proteinuria had no bradykininlike activity in their urine.

Plasma samples from 11 patients and 13 control subjects contained kininase that deactivated 10 μg synthetic bradykinin within 10 min. In each sample, salivary-induced bradykinin was similarly deactivated by the plasma kininase within 12 min. Urinary-induced bradykinin, however, was still present in many of the urine-plasma mixtures after 12 min, but in only two (one CFP and one CS) was there residual bradykinin activity after 22 min. Salivary kininase activity was detected in both patients and control subjects. Inactivation of 10 μg of synthetic bradykinin by 0.5 ml of saliva was much slower than that by plasma kininase and usually required up to 90 min. No kininase activity was detected in urine of patients or control subjects.

Slight kallikrein activity was detected in urine of both groups. Kallikrein measured 1.95 ± 1.7 units in 11 control subjects compared with 1.59 ± 0.42 units in 8 patients. This difference was not significant.

The saliva from 11 patients showed a significantly greater kallikrein content (19.3 ± 13 units) than that from 18 control subjects (7.2 ± 8 units). In patients, however, protein content was greater than in control subjects, and kallikrein activity correlated directly with the total protein content of the saliva ($r = 0.671$; $p < 0.005$). The results obtained from kallikrein assays with saliva suggest the presence of an inhibitor to kallikrein in many of the specimens from CFP and CS, since the formation of bradykinin did not increase with higher concentrations of saliva. Storage of the saliva at 0 or 4° for approximately 1 week resulted in loss of this inhibitory effect in most instances, and a linear relation between the amount of saliva and the resultant bradykinin activity at all concentrations of saliva was observed.

This evaluation of the bradykinin-forming system in plasma, urine and saliva failed to yield any evidence for excessive formation or impaired inactivation of bradykinin; however, mixed saliva of patients with cystic fibrosis of the pancreas was found to contain significantly higher kallikrein activity than that of normal controls. It is likely that this increase in salivary kallikrein accompanies the overall increase of organic constituents known to exist in saliva of patients with this disease. It is postulated that the abnormal secretions that occur in cystic fibrosis of the pancreas may result from excessive cholinergic stimulation in the presence of cholinergic blockade. This would result in an overall increase of organic constituents in the secretion, including kallikrein, and in a low secretory volume.

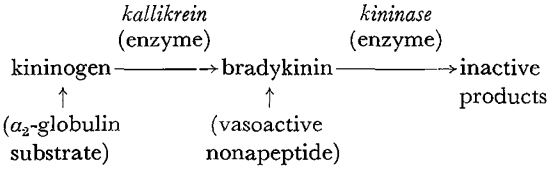
Speculation

The results of this study failed to demonstrate a defect of the bradykinin system that could be responsible for the abnormal secretions associated with cystic fibrosis of the pancreas. It is possible, however, that an abnormality occurs at the cellular level, and further investigation of this system is indicated.

Introduction

Several theories for the pathogenesis of abnormal exocrine secretions in patients with cystic fibrosis of the pancreas (CFP) have been proposed [5]. Major consideration was given first to the possibility that an abnormal mucoprotein exists in the secretions of such patients, but a number of investigators now suggest that dysfunction of secretory stimuli [17] or of ion transport [10, 14] may be the underlying factor. To further investigate the latter possibilities, study of the bradykinin system in patients with this disease was undertaken.

Bradykinin is a biologically active peptide capable of stimulating the contraction of nonvascular smooth muscle and of causing vasodilatation [16]. Three kinins are produced by the action of a peptidase (kallikrein) on plasma α_2-globulin substrate (kininogen) and are then inactivated by further peptidase activity (kininase).



The glandular tissues in which the kinins were first detected are characterized by rapid alterations in blood flow [16]. Such organs include the pancreas and the salivary, lacrimal, and sweat glands, all of which are abnormal in CFP. It is thought that the kinins may regulate the function of these organs by controlling local blood flow [8, 12] and that they may be mediators of electrolyte transport in some tissues [9]. Thus, a study of the bradykinin system is of prime interest in the search for the basic defect in CFP.

The present study is concerned with an evaluation of bradykininlike activity in urine, saliva, and plasma; kininase activity; and the kallikrein content of urine and saliva in CFP and CS [13]. An increased level of kallikrein in the saliva of CFP is described, and a theory for the pathogenesis of the abnormal secretions in CFP is proposed.

Materials and Methods

Specimens of blood, urine, and mixed saliva were obtained from 11 patients with CFP. Four females and seven males ranging in age from 9 to 30 years (mean age, 16.8 years) were studied. Twenty-six control subjects of comparable ages were also studied, although

all tests were not performed on each control. Unless stated otherwise, blood was drawn with plastic syringes into plastic tubes; all specimens were tested immediately. Saliva was collected without stimulation in glass jars.

Bradykinin Assay

A modified Schultz-Dale technique [4] was used for assay of bradykinin activity (fig. 1). Estrus-rat uterus was prepared by injecting 1.25 mg diethylstilbestrol [22] subcutaneously into a 2- to 4-month-old rat of the Sprague-Dawley strain 24 h prior to sacrifice in a CO_2 (dry ice) atmosphere. One horn of the uterus was suspended in the Schultz-Dale apparatus in 25 ml of Tyrode's solution at 37° with constant flow of O_2 gas. Muscular contractions were recorded on a kymograph rotating at 4 mm/min. The muscle preparation was washed three to five times with Tyrode's solution until spontaneous contractions of the muscle ceased. Up to 0.5 ml of a test solution was then pipetted into the 25-ml muscle bath and the resulting contraction recorded. For a measurement of spontaneous 'smooth-muscle stimulating' (bradykininlike) activity in saliva, urine, or plasma, 0.5 ml of each of these fluids was added directly to the muscle bath. Following each assay, the preparation was rinsed two to three times with Tyrode's solution so that a stable base line reappeared. One horn of the uterus usually remained viable for an entire day if kept adequately oxygenated.

Uterine contractions were measured at a point 1 cm (2.5 min) after the onset of contraction from the base line to the relaxation phase of small spiking contractions usually superimposed on the sustained bradykinin contraction (fig. 2). The relaxation phase of the spiking contractions was found to correlate most closely with the concentration of bradykinin; for example, smaller amounts of bradykinin produced rhythmic spikes of greater amplitude, but the lower base line reflected the reduced amount of bradykinin. The degree of contraction was usually expressed as the percentage of maximum contraction obtainable with an excess of synthetic bradykinin (10–20 μg) [21].

Assay of Kallikrein in Urine or Saliva

A supply of pooled human serum was stored frozen for use as kininogen substrate in the kallikrein assay. Dilution of the serum with Tyrode's solution frequently resulted in a contraction caused by the spontaneous activation of serum bradykinin by serum kallikrein. Activation was prevented by routinely shaking the serum substrate with glass beads (0.1 mm diameter; 170 mg/ml of serum) for 5 min at room temperature. This was then incubated at 37° for 30 min to activate and destroy the substrate for plasma kallikrein [15, 20]. The remaining serum (or plasma) kininogen was not subject to activation by blood kallikrein, but was extremely sensitive to the action of kallikrein from saliva or urine.

Kallikrein activity was measured by mixing 0.5 ml of serum substrate with 0.1 ml of a suitable dilution of saliva or urine in 0.1 ml of 0.04 M di-sodium-ethylenediaminetetraacetic acid (EDTA) to inhibit destruction of bradykinin by kininase. The pH of this mixture was 7.5. Following incubation in a test tube for 4 min at 37°, 0.5 ml of the mixture was added to the muscle bath for bradykinin assay. The assay was performed with several dilutions of the test specimen in saline. The height of uterine contraction was converted to percentage of maximum attainable contraction (determined by addition of 10 μg of synthetic bradykinin).

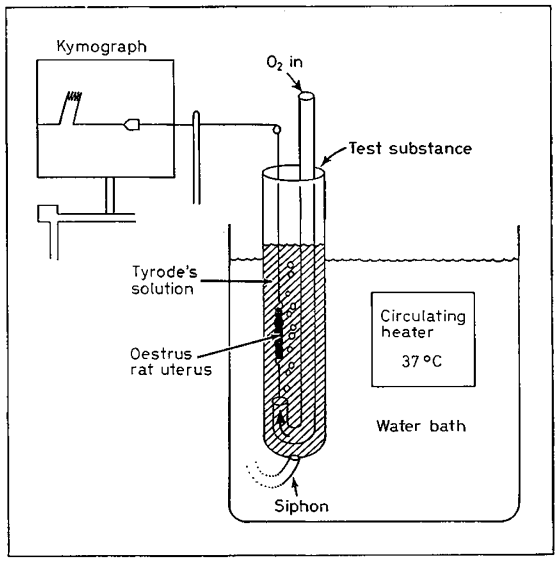


Fig. 1. Diagram of modified Schultz-Dale apparatus.

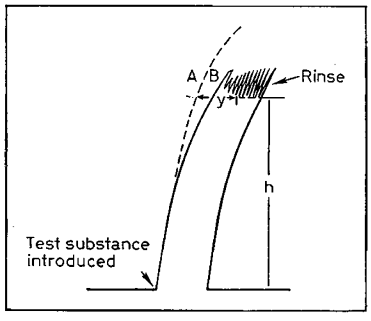


Fig. 2. Method for measuring height of bradykinin-induced contraction of an estrus-rat uterine horn. Height of contraction is measured at point 'Y', 1 cm (2.5 min) after the addition of the test agent, from the base line to the lower limits of the small spiking contractions.

The percentage of maximum contraction was plotted against the log of the dilution of the test specimen, and units of kallikrein activity were selected as the inverse of that dilution interpolated to produce 50% of the maximum muscle concentration (fig. 3).

Evaluation of Kininase Activity

Kininase activity of plasma, urine, and saliva in CFP and CS was determined by incubating 0.5 ml of urine, saliva, or glass-treated plasma with 10 μg of synthetic bradykinin (0.1 ml) plus 0.4 ml of Tyrode's solution (pH 7.4) at 37°. A sample of 0.1 ml of the mixture was tested immediately and at intervals up to 1.5 h.

Kininase activity in plasma of patients was also tested against bradykinin formed by the action of their urine or saliva on their own plasma. This was done by incubating 1.0 ml of plasma with 0.2 ml of urine or saliva plus 0.2 ml of physiological saline instead of EDTA. The mixture (0.5 ml) was then assayed for bradykinin activity following incubation for 2 and 12 min at 37°.

Measurement of Protein Content

Protein content of saliva was measured by a standard biuret technique using crystalline egg white albumin [23] as a standard. Protein content in urine was detected with 3% sulfosalicylic acid reagent.

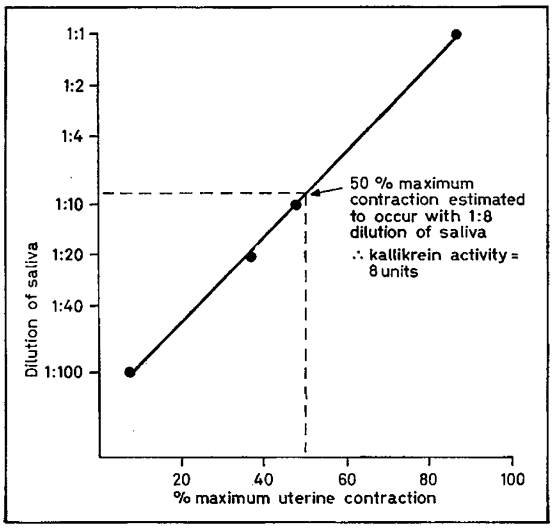


Fig. 3. Method for quantitating kallikrein activity in urine or saliva. Units of activity equal the reciprocal of that dilution (urine or saliva) interpolated to produce 50% of maximum uterine contraction.

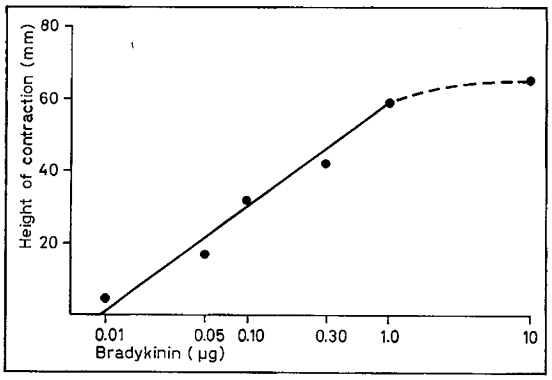


Fig. 4. Standardization curve with synthetic bradykinin. The abscissa indicates amount of bradykinin added to 25 ml of the muscle-bath solution.

Results

Standardization of Bradykinin Assay

The bradykinin assay detected as little as 0.01 μg synthetic bradykinin activity or 4×10^{-4} μg/ml of bath solution (fig. 4). The height of uterine contraction was directly proportional to the logarithmic concentration of bradykinin, and maximum contraction occurred at levels of 1–10 μg. A maximum variation of $\pm 15\%$ was observed with repeated assays of a fixed concentration of synthetic bradykinin.

Bradykininlike Activity in Urine, Saliva, and Plasma

Free bradykininlike activity in urine, saliva, or plasma specimens from 11 patients was the same as that from 21 control subjects (table I). In saliva specimens from CFP and CS no bradykininlike activity was detected, whereas in urine, there was slight activity in approximately 60% of the samples. The frequency with which such activity was detected and the level of activity in urine did not differ significantly between the two groups.

Fresh heparinized plasma of both patients and control subjects developed spontaneous bradykinin activity when it was added to the muscle bath; this type of activity could be prevented by shaking the plasma with glass beads and incubating the specimens at 37° for 30 min. All of the 'glass-shaken' plasma specimens developed normal-appearing bradykinin activity when activated by salivary kallikrein.

The presence of bradykininlike activity in urine was unrelated to proteinuria since none of the patients with activity in urine had detectable proteinuria. Five patients with the nephrotic syndrome and heavy proteinuria had no bradykininlike activity in urine.

Kininase Activity of Plasma, Saliva, and Urine

Plasma samples from 11 patients and 13 control subjects deactivated 10 μg of synthetic bradykinin within a 10-min incubation period. In each sample, salivary-induced bradykinin was similarly deactivated by the plasma kininase within 12 min. Urinary-induced bradykinin, however, was still present in many of the urine-plasma mixtures after 12 min, but in only two (one CFP and one CS) was there residual bradykinin activity after 22 min.

Salivary kininase activity was detected in both patients and control subjects. Inactivation of 10 μg of synthetic bradykinin by 0.5 ml of saliva was much slower than that by plasma kininase and usually required up to 90 min.

No kininase activity was detected in urine of patients or control subjects.

Kallikrein Activity of Urine

Slight kallikrein activity was detected in urine of both groups. Kallikrein measured 1.95 ± 1.7 units in 11 control subjects, compared with 1.59 ± 0.42 units in 8 patients. This difference was not significant.

Kallikrein Activity of Saliva

The saliva from 11 patients showed a significantly greater kallikrein content than that from 18 control subjects (table II). Activity was almost three times greater in saliva of CFP than in that of CS; in patients, however, protein content was greater than in the control subjects, and kallikrein activity correlated directly with the total protein content of the saliva (correlation coefficient = 0.671, n = 25, $p < 0.005$) (fig. 5).

The results obtained from kallikrein assays with saliva suggest the presence of an inhibitor to kallikrein

Table I. Bradykininlike activity in urine and saliva

	Urine		Saliva	
	Controls	CFP	Controls	CFP
No. of patients	21	11	21	11
No. with free bradykinin activity	13 (62%)	7 (64%)	0	0
Mean activity[1]	8.7 ± 6	11.6 ± 10	—	—
Range	(2.8–17.6)	(3.4–33)	—	—
Significance	ns[2]			

[1] Percentage of maximum contraction ±SD.
[2] ns = not significant.

Table II. Relation between kallikrein and protein content of saliva

Patients	Kallikrein, units[1]	p value	Protein content, mg/ml	p value	Kallikrein-protein, ratio	p value
CS[2]						
Mean	7.2 ± 8[3] (n = 18)		1.680 ± 0.35 (n = 13)[4]		5.251 ± 4.51 (n = 13)	
Range	(1.0–34)		(1.1– 2.2)		(0.500–15.454)	
CFP[5]						
Mean	19.3 ± 13 (n = 11)	< 0.02	2.880 ± 1.02 (n = 11)	< 0.001	6.567 ± 3.37 (n = 11)	ns[6]
Range	(1.7–40)		(1.420– 4.650)		(1.197–12.653)	

[1] Inverse of saliva dilution causing 50% of maximum muscle contraction.
[2] CS = control subjects.
[3] ±SD.
[4] Numbers in parentheses indicate number of subjects studied.
[5] CFP = patients with cystic fibrosis of the pancreas.
[6] ns = not significant.

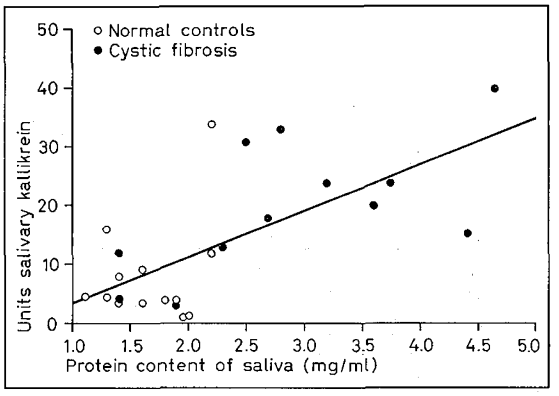


Fig. 5. Relation between kallikrein activity of saliva and the total protein content. Correlation coefficient = 0.671, n = 25, p < 0.005. A regression curve is shown.

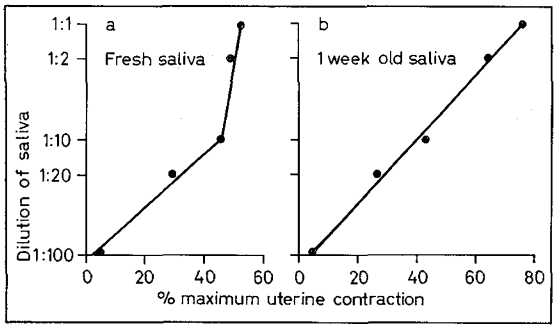
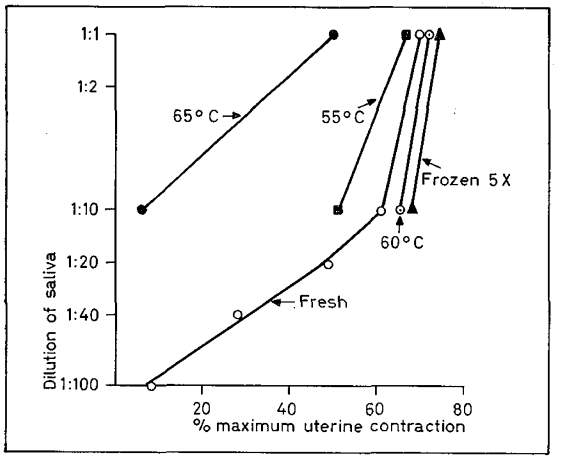


Fig. 6. Comparison of salivary kallikrein assays with fresh specimen and after storage for 1 week at 4°. Presence of kallikrein inhibitor is suggested by lack of linear response in fresh specimen to higher concentrations of saliva (a). Inhibitory effect is lost after storage (b).



in many of the specimens from CFP and CS, since the formation of bradykinin did not increase with higher concentrations of saliva (fig. 6). This effect was not due to limitations of serum substrate, because doubling the amount of serum mixed with saliva did not increase bradykinin production. The inhibitory effect also did not appear to be the result of mechanical limitations of the assay system or inhibition of uterine contractability, inasmuch as a greater degree of uterine contraction could be obtained by adding synthetic bradykinin to the system. It is possible, however, that the inhibitory effect may have been due to inactivation of bradykinin caused by the increased amounts of salivary kininase that were added with increasing amounts of saliva, although the kininase should have been inhibited by the EDTA. Storage of saliva at 0 or 4° for approximately 1 week resulted in loss of this inhibitory effect in most instances, and a linear relation between the amount of saliva and the resultant bradykinin activity at all concentrations of saliva was observed. The apparent inhibition of kallikrein activity was seen mostly with specimens of saliva containing high kallikrein activity.

Neither kallikrein nor its inhibitor was affected by repeating the freezing and thawing process five times (fig. 7). Kallikrein activity in whole saliva was sensitive to heating at 65° for 30 min; it was unaffected by heating at 55 or 60°.

Discussion

This evaluation of the bradykinin-forming system in plasma, urine, and saliva failed to yield any evidence for excessive formation or impaired inactivation of bradykinin; however, mixed saliva of patients with cystic fibrosis of the pancreas was found to contain significantly higher kallikrein activity than that of normal controls. It is doubtful that the increased kallikrein content of saliva is the abnormality underlying the pathogenesis of CFP. It is more likely that this increase in salivary kallikrein accompanies the overall increase of organic constituents shown to exist in saliva of patients with this disease [3]. This latter interpretation is supported by the positive correlation between kallikrein activity and total protein content of saliva, and by the higher protein levels found in saliva of CFP.

Although the unusually high concentration of kallikrein in saliva does not appear to be a primary abnormality in CFP, control mechanisms for the secretion of this enzyme and the potential mechanism for its over-secretion in saliva will be considered.

Fig. 7. Effect of heating and of freezing and thawing on salivary kallikrein activity. Whole saliva was heated at indicated temperatures for 30 min.

The kallikrein contained in saliva does not form bradykinin unless kallikrein is also released into the interstitial fluid of the gland. Since saliva contains no kininogen, bradykinin cannot be formed directly. HILTON and LEWIS [11, 12], however, have shown that neural stimulation of the salivary gland results in release of kallikrein into the interstitial fluid of the gland, where it forms bradykinin from the α_2-globulin contained therein. This bradykinin is subsequently carried away by blood and lymph. These investigators claim that the interstitial bradykinin affects the local vasculature and causes hyperemia by means of vasodilator action. SCHACHTER and BEILENSON [18] have reported conflicting evidence that salivary kallikrein may not play this major role in the rapid vasodilatation that accompanies neural stimulation of the gland; they feel that the vasodilatation is purely cholinergic and not mediated via bradykinin formation. Nevertheless, bradykinin is a potent vasodilator, and any source of excess formation would be expected to produce local hyperemia and possibly systemic vascular effects. The failure of such conditions to occur in CFP suggests that salivary kallikrein does not penetrate interstitially, but is merely contained in high concentration in the salivary exocrine secretion.

HILTON and LEWIS [11, 12] have also shown that kallikrein output from salivary glands of cats can be initiated by either an intraarterial injection of acetylcholine (ACh) or by stimulation of the cholinergic chorda-tympani nerve. Atropine prevents this effect of ACh but has minimal effect on kallikrein output resulting from neural stimulation. The authors showed that atropine can interfere with the ability of the chorda nerve to stimulate saliva formation, but cannot abolish the ability of the nerve to stimulate kallikrein production. Thus, kinin-forming enzyme is released after stimulation in the presence of atropine, even though little saliva is formed. This finding suggests a mechanism whereby a glandular secretion may have high enzyme content but low overall volume, which is seen in CFP and is, namely, excessive neural stimulation of the gland in the presence of an atropine-like inhibitor.

Kallikrein is also found in the pancreas. HILTON and LEWIS [12] have found that large amounts of kinin-forming enzyme were released into a vascular perfusate from the gland when the pancreas was activated by ACh or by pancreozymin. Secretin lacked vasodilator activity and did not give rise to significant release of kinin-forming activity into the perfusate, but it did cause secretion of pancreatic juice with high kinin-forming activity. This resembles the effect on the salivary gland of stimulating the chorda-tympani nerve when salivation is blocked by atropine, that is, high secretory kallikrein concentrations without the release of interstitial kallikrein.

These observations on the mechanisms for control of kallikrein secretion in the salivary glands and pancreas suggest that various derangements of secretory control can affect differently the secretory end product:

If the high concentrations of kallikrein observed in saliva of CFP were due to excessive pancreozymin-like or neural stimulation alone, there should be evidence of increased interstitial kallikrein in the salivary gland with local hyperemia and possibly systemic vascular effects; this has not been reported in CFP.

If the abnormal secretions result from an excessive secretin-type stimulus with normal neural or pancreozymin-like stimulus, the kallikrein concentration of the secretion would be high, with adequate content of water and electrolytes but with low overall organic content. This, too, does not coincide with what has been observed in saliva of CFP.

A deficiency of secretin-like stimulation, or stimulation by the chorda-tympani nerve in the face of cholinergic blockade, however, would result in an overall increase in all organic constituents of the saliva, including kallikrein and in reduced content of water and electrolyte. This best fits the description of saliva in CFP, except that salt content has been found to be inconsistently high. The concept is also consistent with the theory of HADORN *et al.* [10], who postulate that a secretin-type deficiency causes the inadequate fluid and electrolyte content of some secretions and the high salt content of sweat in CFP. Overactivity [1, 2, 6, 7] or dysfunction [17] of the autonomic nervous system has been described, and other investigators have demonstrated an inhibitory factor in blood [19] and sweat [14] of patients with this disease. No attempt, however, has been made to reproduce CFP in an animal model by combining parasympathomimetic stimulation with cholinergic blockade. Such experiments are now underway in our laboratory.

In the present study, except for the increased concentration of kallikrein in the saliva, the bradykinin system was essentially normal. This does not preclude the possibility that bradykinin metabolism may be abnormal at the cellular level, and DI SANT-AGNESE's [5] portent of an abnormality in the bradykinin system in CFP way yet be substantiated.

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23. Nutritional Biochemicals Company, Cleveland, O.
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