

A Zinc Protein Isolated from Human Parotid Saliva

(taste buds/metalloprotein/hypogeusia)

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ABSTRACT A zinc protein has been isolated and purified to apparent homogeneity from subjects with normal taste acuity by gel filtration and ion-exchange chromatography. The protein has a molecular weight of 37,000 and does not appear to have subunits. It is composed of 8% histidine residues and has 2 moles of zinc per mole of protein.

Saliva plays an important role in taste, although its function has not yet been fully elucidated. Patients with xerostomia (decreased or absent saliva) exhibit hypogeusia (decreased taste acuity), which can be correlated with pathological changes in taste bud anatomy (1). Hypogeusia has also been observed in rats in which salivary glands have been extirpated (2). Oral administration of water or saline, with or without other electrolytes, failed to restore taste function toward normal in patients with xerostomia (1); however, treatment with agents that restore salivary function to normal resulted not only in recovery of normal taste acuity but also the re-appearance of normal taste buds (1). Patients with hypogeusia of various etiologies in whom salivary flow rates are normal show pathological changes in taste buds that are similar to those observed in patients with xerostomia (3, 4).

Zinc plays an important role in taste perception (3-7). Some patients with hypogeusia exhibit lower than normal concentrations of zinc in serum (4, 5) and in parotid saliva (8, 9). Administration of zinc to some patients with hypogeusia has normalized serum and parotid zinc levels, taste perception, and taste bud anatomy (3-7). Zinc has also been identified by laser microprobe studies at or near the taste bud in rat circumvallate papilla (5). Although these results suggest a role for zinc in taste, its function has not yet been elaborated.

Many factors influence taste and may relate to the unique properties of taste buds. First, taste buds do not contain blood vessels or lymphatic channels (4). Second, although their cellular components turn over rapidly (4, 10), mitotic figures are only rarely observed in taste bud cells (4). Third, new cells apparently migrate into the taste bud from the areas surrounding it, differentiating into specific taste cells within the bud, under neural, salivary, and perhaps other influences (4). Thus, the manner by which taste buds develop and are nourished has not yet been clearly resolved.

From these physiological, anatomical, and clinical observations of taste perception and taste bud development and from the knowledge that zinc is commonly found associated with a protein in biological fluids, we hypothesized that a zinc protein was a constituent of normal parotid saliva and that its function was related to the growth and nutrition of taste buds. In order to test the first of these two hypotheses, we

sought to find a zinc protein in parotid saliva in subjects with normal taste acuity. In this paper we report on the isolation and chemical properties of a zinc protein that has been purified to apparent homogeneity from human parotid saliva.

MATERIALS AND METHODS

To collect saliva, plastic Lashley cups were placed over Stenson's ducts and salivary flow was stimulated; 100-300 ml of whole parotid saliva was collected from subjects with normal taste acuity. Saliva was lyophilized in acid-washed glass flasks. When needed, the dry product was dissolved in 5 ml of zinc-free distilled water (11) and allowed to stand in ice for 1-3 hr. The reconstituted saliva was centrifuged at $20,000 \times g$ for 30 min at 0° to remove flocculent and insoluble protein. No difference was observed in the chromatographic procedures whether the supernatant was dialyzed against 0.01 M Na phosphate (pH 6.8) or used directly. Smaller amounts of saliva were also collected from other subjects with normal taste acuity and from 47 patients with hypogeusia of various etiologies (4, 8, 9); blood was also collected. Zinc concentrations were determined in both fluids (8, 9, 11).

Water, reagents, and glassware used in all studies were demonstrated to be zinc-free as measured by atomic absorption spectrophotometry (11). Dialysis tubing was soaked in 0.01 M EDTA for 24-72 hr, rinsed, soaked, and stored in zinc-free water until ready for use. Plastic equipment was used whenever possible.

Gel filtration and ion exchange chromatography was carried out with Sephadex G-150 and DEAE-A-Sephadex 50 (Pharmacia) and with carboxymethylcellulose (CM-cellulose) (CM 52, preswollen, Whatman).

Protein in parotid saliva was measured by three spectroscopic methods that reflect distinctive protein properties; absorbance at 215 nm and 280 nm and fluorescence at 340 nm. Absorbance at 280 nm is determined principally by the amount of tyrosine and tryptophan. Intensity of fluorescence at 340 nm is dependent upon tryptophan quantum yield. Absorbance at 215 nm is determined principally by peptide chromophores. We used differences in absorbance between 215 nm and 225 nm (called $\Delta 215$), since this method largely eliminates any absorbance due to turbidity. Emission intensities were measured with a Perkin Elmer Hitachi.

Zinc concentration in all column eluates was measured with an Instrumentation Laboratory 355 flameless sampler attached to a 153 atomic absorption spectrophotometer (8, 9) and calculated by comparison with a standard zinc curve. Since the eluates from the various columns contained different buffer and salt concentrations, the zinc concentrations shown in Figs. 1-3 are only approximate (9). Determination of the ratio of zinc to protein of the purified protein from the CM-cellulose column was made by comparison with a

Abbreviations: CM-cellulose, carboxymethylcellulose; NaDod-SO₄, sodium dodecyl sulfate.

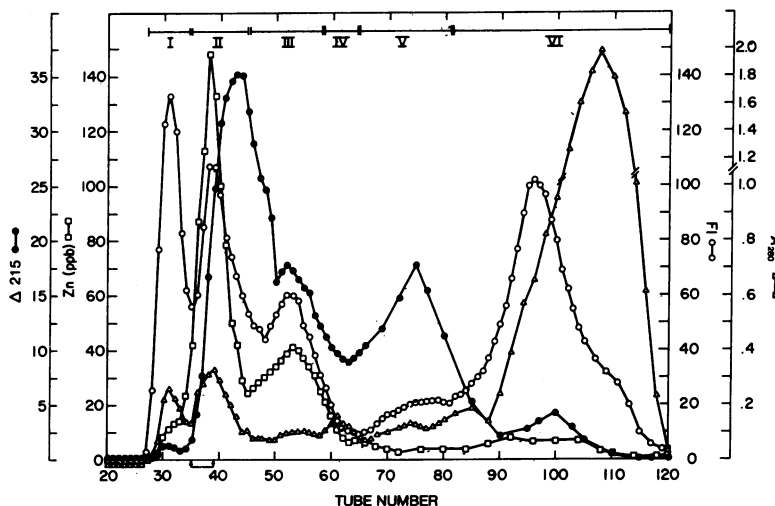


FIG. 1. Sephadex G-150 chromatography of whole parotid saliva (100-ml sample, lyophilized, then dissolved in 5 ml of water). The column was eluted with 0.01 M phosphate buffer (pH 6.8) (4.2 ml per tube). $\Delta 215$, difference in absorbance between 215 nm and 225 nm; Zn concentration (parts per billion) determined with reference to an aqueous standard curve for zinc; Fl, relative fluorescence observed at 340 nm with activation at 280 nm. Six fractions were defined and labeled as I-VI. Over 90% of the applied $\Delta 215$ absorbance was recovered from the column. Arrows on abscissa indicate that the contents of tubes 35-39 were combined and chromatographed on DEAE-A50-Sephadex.

standard zinc curve determined in solutions containing the same concentrations of NaCl and phosphate as the sample. Zinc concentrations were obtained in two dilutions of the purified protein. Concentration of the purified protein was determined by comparing the absorption at 210 nm and 205 nm with that of a group of seven well-known, purified proteins (R. Lippoldt and H. Edelhoch, unpublished observations). The mean absorbance for a 1% solution for these standard proteins was 214 ± 14 and 328 ± 14 (mean \pm standard deviation), respectively, at 210 nm and 205 nm.

Amino-acid analysis was performed on two separate preparations of the purified protein on a Beckman 121 automatic amino-acid analyzer after hydrolysis in 6 M HCl at 108° for 24, 48, and 72 hr in an oxygen-free, nitrogen atmosphere.

Values for serine, threonine, and aspartic acid were corrected for losses by extrapolation to 0 time. Values obtained at 72 hr were used for leucine and proline. For the other amino acids, values were averaged for the three time periods. Methionine and half-cysteine were determined after performic acid oxidation by a modification of the method of Hirs (12). Tryptophan concentration was estimated from the protein absorbance at 280 nm and from the tyrosine content as determined from the amino-acid analysis. Molar extinction coefficient values of 5500 and 1200 were used for tryptophanyl and tyrosyl absorbance at 280 nm, respectively (13).

The molecular weight was obtained by equilibrium sedimentation in a Spinco model E ultracentrifuge with scanner optics. The method of meniscus depletion was used with a

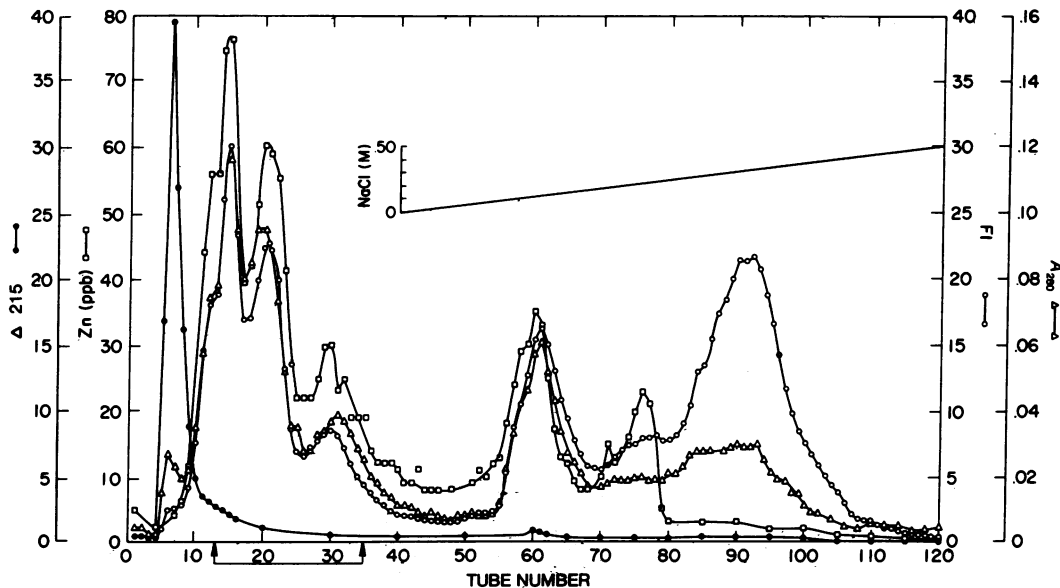


FIG. 2. DEAE-A50-Sephadex chromatography of the contents of tubes 35-39 from Sephadex G-150 (5.0 ml per tube). Column was eluted initially (tubes 1-41) with 0.01 M phosphate buffer (pH 6.8) and then with a continuous NaCl gradient (0.0-0.50 M) in the same buffer, total volume 500 ml (tubes 41-120). Ordinate symbols are the same as those in Fig. 1. About 85% of the applied $\Delta 215$ absorbance was recovered from this column. The contents of tubes 14-34 (arrows on abscissa) were combined, and chromatographed on the CM-cellulose column.

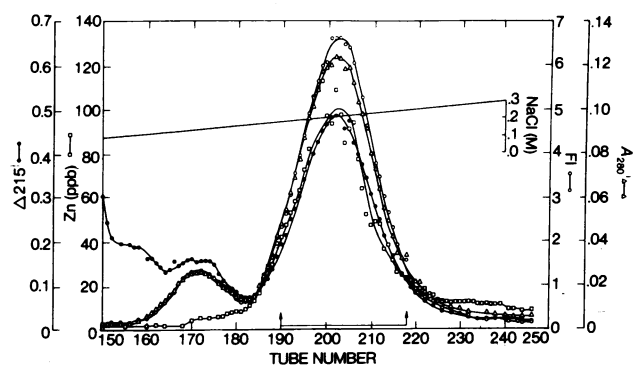


Fig. 3. CM-cellulose chromatography of the contents of tubes 14-34 from DEAE-A50-Sephadex column (1.0 ml per tube). Column was eluted initially (tubes 1-86) with 5 mM phosphate buffer (pH 5.9), and then with a continuous NaCl gradient (0.0-0.3 M) in the same buffer, total volume 170 ml (tubes 91-260). For convenience, only results of tubes 150-248 are shown. Ordinate symbols are the same as those in Fig. 1. All $\Delta 215$ absorbance applied was recovered from this column.

4-mm column height. The same molecular weight was obtained after 72 and 96 hr, indicating that equilibrium was reached. Solutions were thoroughly dialyzed against either 0.1 M NaCl or 6.0 M guanidinium hydrochloride before centrifugation.

Polyacrylamide gel electrophoresis was performed in continuous buffer systems as described by Hjerten (14). Protein was stained with Coomassie brilliant blue R 250 (15).

Neutral sugar content of the purified protein was determined by the phenol sulfuric acid method (16), with galactose as a standard.

RESULTS

Chromatography

Sephadex G-150 Column. The lyophilized parotid saliva dissolved in 0.10 M phosphate buffer (pH 6.8) (about 800 mg of protein) was applied on a Sephadex G-150 column (2.5×90 -cm) which was equilibrated with 0.10 M phosphate buffer (pH 6.8). Elution with the same buffer gave the patterns shown in Fig. 1*. Elution profiles obtained by the three methods of protein analysis are quite distinct since different protein properties are measured by each technique (see *Materials and Methods*). The $\Delta 215$ absorbance profile was divided into six major fractions, which are labeled I to VI in Fig. 1. A more comprehensive description of total fractionation of the non-zinc-containing proteins of saliva will be presented elsewhere. Fraction I appeared in the solvent front and although highly fluorescent, showed very little $\Delta 215$ absorbance. Fraction II was divided into two parts (A, tubes 35-39; B, tubes 40-45), since the protein in the first part (IIA) contained a much higher Zn/ $\Delta 215$ ratio than that in the second part (IIB). Fraction IIA was lyophilized; when needed it was dissolved in 5 ml of zinc-free water and dialyzed overnight against 0.01 M phosphate buffer (pH 6.8).

* This parotid saliva came from a woman from whom collections were obtained on 24 separate occasions. Saliva from about two-thirds of these collections were eluted on the same Sephadex G-150 column, and the major features of the patterns shown in Fig. 1 were reproduced. The basic features of this pattern were observed in the saliva obtained from other subjects with normal taste acuity, although some differences existed. In other subjects with normal taste acuity the zinc was distributed over a wider range, frequently extending into fraction III.

TABLE 1. Specific activities and yield of parotid zinc protein

Substance	Tubes	Specific activity, Zn (ppb)/ $\Delta 215$	Specific activity, Zn (ppb)/ $A_{280 \text{ nm}}$	Yield* of parotid zinc protein, %
Whole parotid saliva		1.1	5.2	
Sephadex G-150 column	35-39	9.4	470	5.9
DEAE-A50-Sephadex column†	14-34	63	630	0.4
CM-cellulose column	190-218	210	800	0.06

* Yield is defined as the relative amount of absorbance ($\Delta 215$) in the tubes selected from the specific column compared to the amount of absorbance ($\Delta 215$) in the original, whole parotid saliva.

† The second zinc peak was used to avoid the overlap with the strong $\Delta 215$ peak preceding the zinc peaks.

DEAE-A50-Sephadex Column. The dialyzed solution of fraction IIA (about 130 mg of protein collected from four Sephadex G-150 column runs) was placed on a DEAE-A50-Sephadex column (1.5×24 -cm) equilibrated with the same buffer. Tubes 1-40 were obtained by elution with the equilibration buffer (Fig. 2), whereas tubes 41-120 resulted from the linear gradient of NaCl (0-0.5 M) in the same buffer. The major separation of the $\Delta 215$ absorbance peak from the zinc, fluorescence, and 280 nm absorbance peaks occurs before the NaCl gradient was started. Three zinc peaks (two major and one minor) were resolved that resembled each other rather closely since the ratio of Zn/280 nm/fluorescence in each peak was similar. Elution with the NaCl gradient resolved two additional zinc peaks, representing about 30% of the zinc on the column; however, the zinc/protein ratios of these peaks were lower than those obtained with the buffer alone, and they were not used in subsequent analyses. The contents of tubes 14-34 (Fig. 2) were pooled and lyophilized. The product was dissolved in water and dialyzed for 24 hr against 5 mM phosphate buffer (pH 5.9).

CM-Cellulose Column. The dialyzed solution of tubes 14-34 (about 10 mg of protein) was placed on a CM-cellulose column (1.5×12 -cm) equilibrated with 5 mM phosphate buffer (pH 5.9). Tubes 1-86 were obtained by elution with buffer alone, whereas tubes 87-240 resulted from a linear gradient of NaCl (0-0.30 M) in 5 mM phosphate buffer (pH 5.9) (Fig. 3). Two major protein peaks showing $\Delta 215$ absorbance without significant 280 nm absorbance, fluorescence, or zinc were resolved in tubes 1-86. The zinc was concentrated largely in a single peak centered at tube 202. The ratio of zinc to protein in this peak was constant between tubes 190 and 218 and independent of the parameter used to evaluate protein concentration (i.e., $\Delta 215$, absorbance at 280 nm, or fluorescence). Therefore, the protein in these tubes must be quite homogeneous if it is not a single species.

The specific activity of the parotid zinc protein (Zn/ $\Delta 215$ or Zn/280 nm), as obtained from the maximum value in the zinc peaks in the three chromatographic columns, is shown in Table 1. It is evident that elimination of contaminating 280 nm absorbing material occurs mainly on the Sephadex G-150 column, whereas $\Delta 215$ contaminating material is eliminated largely on the DEAE-A50-Sephadex column.

There is obviously a great disparity in the $\Delta 215/280$ nm absorbance ratio of many of the proteins. The value used to calculate the specific activity of the starting solution was the zinc concentration and the $\Delta 215$ absorbance of the whole parotid saliva. The results indicate that a 200-fold purification of the zinc protein (Table 1) was accomplished by the three chromatographic procedures. Yields were necessarily low since they were sacrificed for purity in selecting only tubes with the highest zinc/protein ratios from the Sephadex G-150 column for use in the further purification on the DEAE-A50-Sephadex columns.

Molecular characterization

Polyacrylamide Gel Electrophoresis. Electrophoresis of the purified parotid zinc protein at pH 7.2 in 0.05 M phosphate buffer (Fig. 4, gel C) revealed one broad band, while at pH 8.9 in 0.05 M Tris, 0.05 M glycine buffer (Fig. 4, gel B) one major band and about eight more slowly migrating minor components were observed. Electrophoresis at pH 7.2 in 0.1% sodium dodecyl sulfate (NaDodSO₄), 0.1 M sodium phosphate (Fig. 4, gel A1) showed one major band and one faint, more slowly migrating band. The molecular weights of major and minor components in NaDodSO₄ gel, determined by comparison with appropriate standards, were 44,000 and 84,000, respectively. After reduction with 2-mercaptoethanol (Fig. 4, gel A2), electrophoresis in NaDodSO₄ showed no significant change in the migration of the major band although some diffuse staining was evident on either side of the major band. The presence of one predominant band at pH 7.2 in either aqueous or NaDodSO₄ solutions, and the pattern of the minor bands observed at pH 8.9 suggest these minor bands are polymers of the native protein, as observed frequently with serum albumin.

Sedimentation Equilibrium. The molecular weight of the purified zinc protein was measured by sedimentation equilibrium in 0.1 M phosphate buffer (pH 6.8). No difference in molecular weight was found between 72 and 96 hr of centrifugation at 28,000 rpm at 25°. A linear dependence of $\ln c$ against R^2 was observed (Fig. 5). From the slope of the line a molecular weight of 37,000 was calculated by the Svedberg equation when 0.723 was used for the partial specific volume (\bar{v}) computed from the amino-acid composition.

Equilibrium centrifugation in 6.0 M guanidium·HCl also showed a linear dependence of $\ln c$ against R^2 and gave a molecular weight of 35,000 with an assumed \bar{v} of 0.720. Since \bar{v} differs slightly in 6.0 M guanidinium·HCl and in water with some proteins, there is an uncertainty in the calculated molecular weight (17). However, this molecule does not dissociate in the presence of guanidinium·HCl.

Zinc Content. Determination of the number of moles of zinc per mole of protein at two protein concentrations (see *Materials and Methods*) gave a ratio of $2.1 \pm 2\%$ (mean \pm standard deviation) and $2.2 \pm 2\%$ when the protein concentration was determined at 210 nm and 205 nm, respectively, and a molecular weight of 37,000 was used.

Composition. The amino-acid composition of the purified zinc protein is shown in Table 2. The protein contains a rather large amount of histidine, representing more than 8% of the residues. No carbohydrate was detected in the purified zinc protein.

Zinc levels in blood and parotid saliva in man

Both serum and parotid zinc concentrations in subjects with normal taste acuity were higher than in patients with hypo-

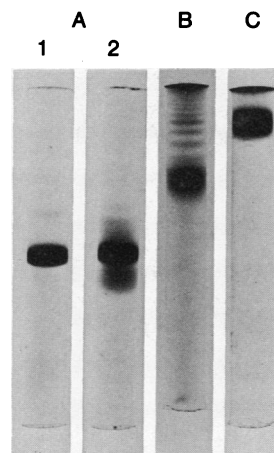


FIG. 4. Polyacrylamide gel electrophoresis of purified parotid zinc protein. The anode is at the bottom of the gels. (A) Electrophoresis buffer, 0.1 M sodium phosphate, 0.1% NaDodSO₄, pH 7.2; gel concentration: T = 4, C = 4. Electrophoresis was carried out at 20° and 8 mA per gel for 3 1/4 hr, with 25 μ g of protein per gel. In gel A2 the protein was reduced with 0.29 M 2-mercaptoethanol at 37° for 1 hr. (B) Electrophoresis buffer, 0.05 M Tris, 0.05 M glycine, pH 8.9; gel concentration: T = 7, C = 4. Electrophoresis was carried out at 28° and 2.5 mA per gel for 1 3/4 hr, with 10 μ g of protein. (C) Electrophoresis buffer, 0.05 M sodium phosphate, pH 7.2; gel concentration: T = 4, C = 4. Electrophoresis was carried out at 20° and 8 mA per gel for 2 1/2 hr with 30 μ g of protein.

geusia (Table 3). In normal subjects the ratio of parotid zinc to serum zinc is 0.5×10^{-3} , whereas in patients with hypo-geusia this ratio is significantly lower, 0.1×10^{-3} . A more profound decrease of zinc occurred in saliva than in serum (Table 3).

DISCUSSION

The results indicate that a zinc protein is a normal constituent of parotid saliva in subjects with normal taste acuity. Chromatography on three columns resulted in the isolation of a zinc protein that appears homogeneous. Homogeneity was indicated by (a) the presence of one band by gel electrophoresis in phosphate buffer, with and without NaDodSO₄, (b) a symmetrical peak on the CM-cellulose column in which

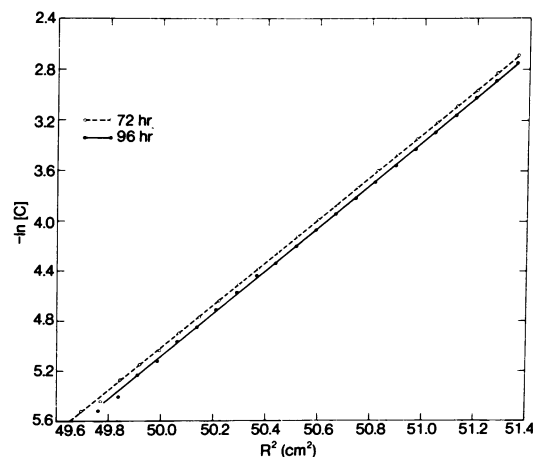


Fig. 5. Sedimentation equilibrium of parotid zinc protein in 0.1 M NaCl-0.01 M phosphate buffer (pH 6.8). The square of the radial distance (R^2) is plotted against the negative logarithm of the protein concentration ($-\ln c$) in grams per 100 ml.

TABLE 2. Amino-acid composition of human parotid zinc protein

Amino acid	Residues/37000 g of protein*	Assumed integers
Lysine	12.3	12
Histidine	20.3	20
Arginine	15.0	15
Aspartic acid	37.3	37
Threonine	24.1	24
Serine	22.0	22
Glutamic acid	35.2	35
Proline	19.0	19
Glycine	23.3	23
Alanine	17.0	17
Valine	23.9	24
Half-cysteine	1.6	2
Methionine	3.7	4
Isoleucine	12.9	13
Leucine	24.0	24
Tyrosine	19.0	19
Phenylalanine	9.3	9
Tryptophan†	4.9	5
Total		324

* Corrected for losses based upon a molecular weight of 37,000 (see *Materials and Methods*).

† Estimated from protein absorbance at 280 nm and from the tyrosine content as determined from the amino-acid analysis. For details, see *Materials and Methods*.

the ratio of zinc to any of the three protein parameters was constant across the peak, and (c) the linearity of the plot of $\ln c$ against R^2 in sedimentation equilibrium experiments. It should be noted that the single, major zinc peak resolved on the CM-cellulose column was derived from the three different zinc peaks observed in the DEAE-A50-Sephadex column. However, gel electrophoresis of each of these peaks from the DEAE-A50-Sephadex column in 0.10 M NaDodSO₄, 0.10 M phosphate, pH 7.2, gave identical patterns with one major band. The differences in elution volumes of these peaks could represent minor differences in the degree of amidation of the aspartic and glutamic acid residues since the peaks were resolved on DEAE-Sephadex but not on CM-cellulose columns. A minor protein peak (tubes 160-189) was also eluted from the CM-cellulose column just ahead of the major zinc protein. The ratios of zinc to the various protein parameters in this minor peak differed from that of the major peak.

The chemical and molecular characteristics of this protein do not closely resemble those of any other zinc protein previously described (18).

Results of physiological studies also indicate the presence of the zinc protein in parotid saliva. After intravenous injection of ⁶⁵Zn to the woman in whom 24 parotid saliva samples were collected (see *Materials and Methods*), ⁶⁵Zn appeared in the parotid saliva within 20 min and continued to rise for 6-8 hr while the blood level was rapidly falling, before leveling off. Fractionation of this saliva on the Sephadex G-150 column revealed a ⁶⁵Zn peak that coincided with that of fraction II, and the ratio of ⁶⁵Zn to unlabeled zinc was constant across fraction IIA. This same result was obtained in 15 separate studies from saliva collected from this patient over a period of 4 months. These results suggest that zinc is rapidly incorporated into the zinc protein *in vivo*.

TABLE 3. Serum and parotid zinc levels in normal subjects and in patients with hypogeusia

Condition	Serum zinc, ppm	Parotid zinc, ppb
Normal subjects	96 ± 2* (150)	51 ± 3 (34)
Patients with hypogeusia†	77 ± 2‡ (125)	10 ± 1‡ (47)

* Mean ± 1 SEM. Number of subjects is given in parentheses.

† Related to several etiological factors (4).

‡ $P < 0.001$ with respect to normal, Student's *t*-test.

The function of the parotid zinc protein in taste is not clearly established. However, its taste function is suggested by lower than normal levels of salivary zinc found in patients with hypogeusia (8, 9) and by demonstration of significantly lower values of Zn/Δ215 in fraction II in patients with hypogeusia (8 ± 1, mean ± 1 SEM) compared to normal subjects (25 ± 3) when chromatographed on Sephadex G-150.

This zinc-containing protein has unique chemical and molecular characteristics. Since this protein appears to be related to the taste process, we suggest that it be called gustin.

We thank Drs. K. Piez and H. Metzger and Mr. G. Hawkins and G. Poy for performing the amino-acid analyses; Dr. Robert Wolf and Mr. Charles Mueller for their assistance; and Drs. Louis Sokoloff and Charles Kennedy for their thoughtful advice.

We dedicate this paper to Dr. Dan F. Bradley. We acknowledge our debt to his pioneering efforts in this field and recognize that the present work could not have been achieved without his influence. We deeply regret that he did not live to see the results of his early labor.

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