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Purification and Properties of Dentinal Fluid Transport Stimulating Substance from Bovine Parotid Glands[1]

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A substance that stimulates dentinal fluid transport (DFT) through the dentinal tubules in the molar teeth of rats was found in bovine parotid glands. This DFT-stimulating substance was purified by aqueous extraction, isoelectric precipitation, ultrafiltration, gel filtration on Sephadex G-75 and chromatofocusing. By these procedures, 37 mg of purified preparation was obtained from 1 kg of bovine parotid gland. The purified preparation showed a single band on both disc polyacrylamide gel electrophoresis and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. A dose of 10 μg of the purified DFT-stimulating substance per kg of body weight was positive in the bioassay for DFT-stimulating activity, as indicated by the migration of a fluorescent dye through the dentinal tubules in the molar teeth of rats.

The molecular weight of DFT-stimulating substance was estimated to be 30000 by both gel filtration on Sephadex G-75 and SDS–polyacrylamide gel electrophoresis. The isoelectric point was found to be pH 6.0 by isoelectric focusing. This active substance was composed of a total of 263 amino acid residues per molecule, had a maximum absorption wavelength (λ_{max}) of 280 nm and an extinction coefficient ($E_{1\,cm}^{1\%}$) at 280 nm of 15.4, contained less than 1% carbohydrate, and appeared to consist of a single polypeptide chain.

Keywords——dentinal fluid transport (DFT); DFT-stimulating activity; fluorescence movement; bovine parotid gland; protein purification; chromatofocusing; DFT-stimulating substance property

Steinman *et al.* demonstrated the existence of the transport of fluid through the hard structures of teeth, and it has been suggested that the first demonstrable alteration in the tooth before dental caries occurs is a marked decrease in fluid transport in the dentin of rat molars[2] and that the rate of fluid transport is related to the incidence of dental caries.[3,4] Furthermore, hypothalamic and parotid tissue extracts have both been shown to stimulate fluid transport through the dentinal tubules in the molar teeth of rats, but hypothalamic extract was effective only when the parotid glands were intact, and the stimulation of fluid transport could not be observed in sialoadenectomized rats. On the basis of these results, Steinman and Leonora suggested that the hormonal stimulation of dentinal fluid transport (DFT) is directly dependent on the parotid glands and that a hypothalamic-parotid gland endocrine axis exists.[5]

The DFT-stimulating substance in parotid glands was named parotid hormone by Steinman and Leonora,[5] and in 1980, this active substance was purified from porcine parotid glands and characterized.[6] It has been shown that this active substance contains 73.3% glycine and proline among the total residues and has a molecular weight of 8100. The partial N-terminal 28 amino acid sequence was also determined. As evidence of the presence of a DFT-stimulating substance in bovine parotid glands has been obtained, we attempted to purify this active substance and to characterize its physicochemical properties.

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Materials and Methods

Materials——Sephadex G-75, chromatofocusing PBE 94 exchanger gel and Polybuffer 96 were purchased from Pharmacia Fine Chemicals. Hollow fiber cartridges H10P100 and H10P5, and Diaflo membrane YM5 were purchased from Amicon Corp. The standard protein for the method of Lowry *et al.*, four times recrystallized bovine serum albumin, was a product of ICN Nutritional Biochemicals Corp. Marker proteins for molecular weight determination, bovine serum albumin, ovalbumin, chymotrypsinogen A and cytochrome c were purchased from Boehringer Mannheim GmbH and bovine pancreas insulin was from Calbiochem. Corp. Isoelectric point markers were products of Oriental Yeast Co. Carrier Ampholite was obtained from LKB-Produkter. Coomassie brilliant blue R-250 was purchased from Nakarai Chemicals. Dowex 50W X-4 was purchased from The Dow Chemical Co. Acriflavine hydrochloride was from Aldrich Chemical Co., and Freund's complete adjuvant from Nakarai Chemicals. Other chemicals were of analytical reagent grade from commercial sources.

Animals——Three-week-old Sprague-Dawley male rats were obtained from Charles River Japan Inc. and fed a pellet diet for one week before use.

Determination of Protein——Protein was determined by the method of Lowry *et al.*,[7] using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis——Analytical disc polyacrylamide gel electrophoresis was carried out on 10% gel with 0.05 M Tris-glycinate buffer, pH 8.3, by a modification of the method of Davis.[8] Electrophoresis was performed at a constant current of 3 mA per tube for 1 h. After electrophoresis, the gel was stained for 1 h with 0.25% Coomassie brilliant blue R-250 solution.

Estimation of Molecular Weight——The molecular weight was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and by gel filtration at 4 °C on a Sephadex G-75 column.

SDS-polyacrylamide gel electrophoresis was carried out on 10% gel containing 0.1% SDS in 0.1 M sodium phosphate buffer, pH 7.2, by a modification of the method of Weber and Osborn.[9] To estimate the molecular weight, standard proteins [bovine serum albumin (66000), ovalbumin (45000), chymotrypsinogen A (25700), cytochrome c (12400) and insulin (6000)] were run under the same conditions. Samples and standard proteins were treated at 95 °C for 5 min in 0.01 M phosphate buffer, pH 7.2, containing 1% SDS in the presence or absence of 2% 2-mercaptoethanol. Electrophoresis was performed at a constant current of 8 mA per tube for 4 h. After electrophoresis, the gel was stained for 1 h with 0.25% Coomassie brilliant blue R-250 solution.

Gel filtration was performed on a Sephadex G-75 column (2.5 × 97 cm) according to the method of Andrews,[10] using standard proteins of known molecular weight (bovine serum albumin, ovalbumin, chymotrypsinogen A and cytochrome c). Elution was performed with 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl at a flow rate of 12 ml/h.

Isoelectric Focusing——Isoelectric focusing was carried out in a column (5 × 80 mm) on 5% polyacrylamide gel using the Carrier Ampholine system as described by Wrigley.[11] The pH range of Carrier Ampholyte was 3.5—10.0 and electrophoresis was performed for 12 h at 200 V (constant voltage) at 4 °C. As visible pI marker proteins, cytochrome c and acetylated cytochrome c were used. After isoelectric focusing, one gel was stained with 0.25% Coomassie brilliant blue R-250 solution. The other gel was cut into 3 mm slices and the pH of the water extract of each slice was measured.

Amino Acid Analysis——Before amino acid analysis, samples were dried under reduced pressure over P_2O_5. The dried samples were hydrolyzed with constant-boiling 6 N HCl containing 0.2% phenol at 110 °C in sealed tubes under a vacuum for 24 and 72 h. After evaporation of the hydrolysate, the amino acid composition was analyzed on an amino acid analyzer, according to the method of Spackman *et al.*[12] Tryptophan was determined by the spectrophotometric method of Edelhoch.[13] Half-cystine was determined from an 18 h hydrolysate after performic acid oxidation.

Carbohydrate Analysis——Hexose content was measured by the phenol-sulfuric acid method,[14] using D-glucose as a standard. For the determination of hexosamine, hydrolysis was carried out in 4 N HCl at 100 °C for 6 h. After evaporation of the hydrolysate, the sample was dissolved in distilled water and passed through a column of Dowex 50W X-4 (H^+ form). The hexosamine was eluted with 2 N HCl as described by Boas[15] and adjusted again to 0.1 N HCl. Hexosamine content was measured by the Elson-Morgan method,[16] using a standard mixture containing equimolar amounts of D-glucosamine and D-galactosamine. For the determination of sialic acid, hydrolysis was carried out in 0.1 N H_2SO_4 at 80 °C for 1 h. The hydrolysate was measured by the periodate-resorcinol method,[17] using *N*-acetylneuraminic acid as a standard.

Preparation of Antiserum to DFT-Stimulating Substance——A sample of 0.5 mg of the purified DFT-stimulating substance in 1 ml of 0.9% NaCl was emulsified with 1.25 ml of complete Freund's adjuvant for each injection. The emulsion was injected into several intracutaneous sites in a rabbit. The injection was repeated four times at two-week intervals. Two weeks after the last injection, the serum was prepared and used as antiserum to DFT-stimulating substance.

Immunodiffusion and Immunoelectrophoresis——Double immunodiffusion was carried out on plates of 1.2% agar gel by the method of Ouchterlony.[18] After 10 μl of the DFT-stimulating substance antiserum had been applied to one well of an agar gel, 10 μl of samples were applied to the other wells. The gel was incubated for 20 h in a humidified

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chamber at room temperature.

Immunoelectrophoresis in 1.2% agar gel was carried out with Veronal buffer, ionic strength $\mu=0.1$, pH 8.6. Samples of 2 μl were applied to wells and electrophoresis was performed at a constant voltage of 250 V for 1 h. The antiserum to DFT-stimulating substance was poured into the trough and the gel was incubated for 20 h in a humidified chamber at room temperature.

Immunoassay of DFT-Stimulating Substance——Single radial immunodiffusion (SRID) was carried out on plates of 1.2% agar gel by the method of Mancini *et al.*[19] Samples and standard proteins in a volume of 5 μl were applied to wells and immunoreaction was performed at 37 °C for 44 h.

Bioassay of DFT-Stimulating Substance——DFT activity was assayed in terms of the ability to stimulate fluid transport, as indicated by the migration of a fluorescent dye through the dentinal tubules. Four-week-old Sprague–Dawley male rats were intraperitoneally anesthetized with sodium pentobarbital in a dose of 30 mg per kg. After the anesthetic had become effective, the sample dissolved in physiological saline was intravenously injected at a dose of 1.0 ml per kg. The control rats were injected with physiological saline. An intraperitoneal injection of a fluorescent dye, acriflavine hydrochloride dissolved in distilled water, was given at a dose of 50 mg per kg 10 min after the infusion of the sample. At 16 min after the fluorescent dye had been given, the rats were decapitated, the upper jaws were quickly removed (in less than 1 min) and immediately frozen. The maxillary molars were sectioned with a microtome under freezing to obtain sagittal frozen sections through the middle of the occlusal surface of the molar teeth; molar sections were cut to about 100 microns thickness. The molar sections were observed with an incident illumination-type fluorescence microscope at 40 magnifications (main excitation wavelength 495 nm), and the movement of acriflavine in molar teeth was observed. The degree of fluid movement to each molar tooth was determined by assigning a value of 0 to 6 with reference to the method for scoring dental caries[20,21]: (value 0) pulp only, (value 2) middle dentinal, (value 4) extensive dentinal, (value 6) up to enamel. If the permeation of fluorescence differed in extent among the molar sections, an intermediate value (1, 3 and 5) was used as the fluid movement value. If the fluorescent dye permeated into the dentin, the dentinal tubules showed a brilliant green fluorescence and the dentino-enamel junction appeared clearly. The fluid movement value of fluorescence was averaged among five rats. The significance of the difference between the mean values of the sample group and control group was calculated by means of the *t*-test. When the values of samples showed a difference at below the 5% level of significance, the sample injected was considered to be effective.

Results

Purification of DFT-Stimulating Substance

All purification procedures were carried out at 4 °C.

Step 1. Preparation of Acetone-Dried Powder——Fresh bovine parotid glands (2.5 kg) were finely ground and stirred with 25 l of acetone for 8 h. The acetone was then removed and replaced with fresh acetone. The same operation was repeated five times to remove fat from the glands. The defatted glands were recovered by filtration and dried to yield 500 g of acetone-dried power.

Step 2. Isoelectric Precipitation——To 500 g of acetone-dried powder, 10 l of distilled water was added, and the mixture was adjusted to pH 7.0 with 1 N NaOH and then homogenized for 2 h. The residue was recovered by filtration and re-homogenized. The combined filtrates were adjusted to pH 5.0 with 1 N HCl, stirred for 1 h and allowed to stand overnight. The resulting precipitate was removed by filtration and the filtered pH 5.0 supernatant was again adjusted to pH 7.0 with 1 N NaOH.

Step 3. Ultrafiltration——The neutralized pH 5.0 supernatant was filtered through a hollow fiber cartridge H10P100 (100000 M_r cut-off) with Amicon DC10J type equipment. The obtained filtrate was concentrated to 1 l using a hollow fiber cartridge H10P5 (5000 M_r cut-off), the low molecular weight substances were removed by distilled water exchange and the crude ultrafiltrate was obtained.

Step 4. Gel Filtration on a Sephadex G-75 Column——The active substance was precipitated by adding ammonium sulfate to 80% saturation. The precipitate formed was recovered by centrifugation, then dissolved in 0.1 M ammonium acetate buffer, pH 5.0, and the insoluble material was removed by centrifugation. The solution was passed through a column (10 × 90 cm) of Sephadex G-75 equilibrated with 0.1 M ammonium acetate buffer, pH 5.0, and eluted with the same buffer (Fig. 1A). There were four peaks of absorbance and the activity

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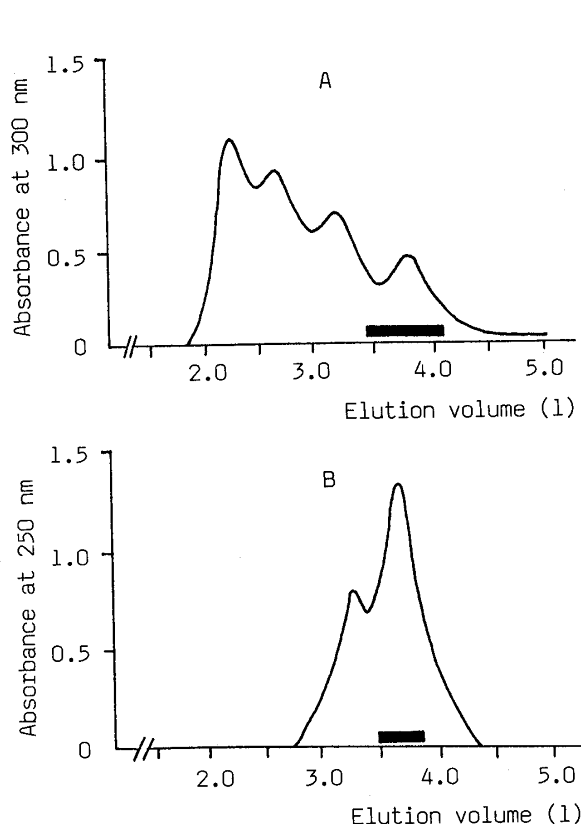

Fig. 1. Gel Filtration on a Sephadex G-75 Column

(A) 1st Sephadex G-75 column. Sample: ultrafiltrate. Column size: 10×90 cm. Eluent: 0.1 M ammonium acetate buffer, pH 5.0. Flow rate: 120 ml/h.

(B) 2nd Sephadex G-75 column. The 1st Sephadex G-75 fraction was rechromatographed under the same conditions as described in (A).

The active fractions indicated by a solid bar were pooled.

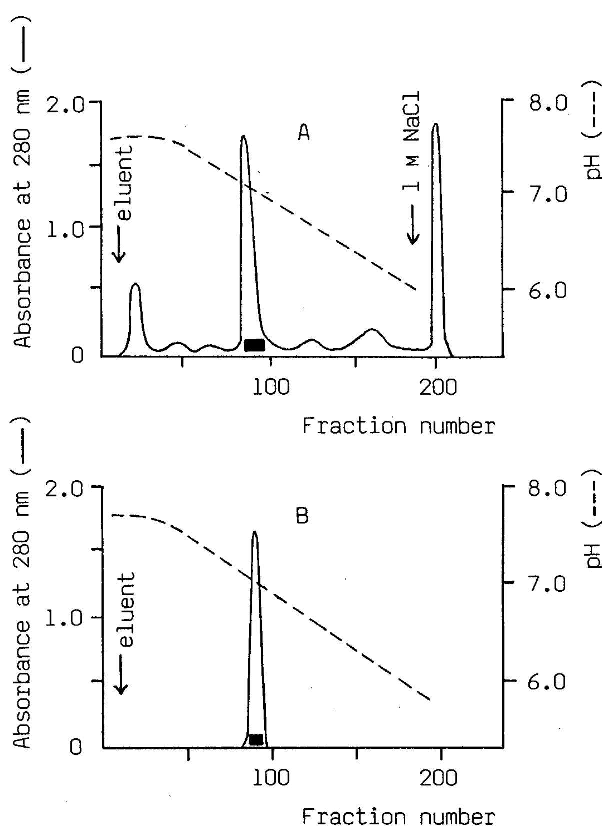

Fig. 2. Chromatofocusing on a PBE 94 Gel Column

(A) 1st chromatofocusing column. Sample: 2nd Sephadex G-75 fraction. Column size: 3.2×43.5 cm. Starting buffer: 0.025 M imidazole–acetic acid, pH 7.5. Eluent: polybuffer 96 (dilution factor 1 : 15)–acetic acid, pH 6.0. Flow rate: 180 ml/h. Fraction: 30 ml/tube.

(B) 2nd chromatofocusing column. The 1st chromatofocusing fraction was rechromatographed under the same conditions as described in (A). Column size: 2.5×50.9 cm. Flow rate: 120 ml/h. Fraction: 20 ml/tube.

The active fractions indicated by a solid bar were pooled.

was present in the fourth peak (elution volume 3.45 to 4.10 l). This active fraction was pooled and concentrated by ammonium sulfate precipitation at 80% saturation. The concentrated solution was again fractionated by a 2nd gel filtration on the same Sephadex G-75 column (Fig. 1B). There were two peaks of absorbance and the activity was present in the second peak (elution volume 3.50 to 3.85 l).

Step 5. Chromatofocusing on a PBE 94 Gel Column——The active 2nd Sephadex G-75 fraction was concentrated by ultrafiltration using a Diaflow YM5 membrane (5000 M_r cut-off) and buffered with 0.025 M imidazole–acetic acid buffer, pH 7.5. This sample was applied to a column (3.2×43.5 cm) of PBE 94 exchanger gel equilibrated with the same buffer and eluted with Polybuffer 96 (dilution factor 1 : 15)–acetic acid, pH 6.0 (Fig. 2A). The main peak, which showed biological activity, was eluted at approximately pH 7.0. The peak fraction, corresponding to fractions 84—92, was pooled and precipitated by adding ammonium sulfate to 80% saturation. The precipitate was recovered by centrifugation, and washed with 80% saturated ammonium sulfate solution five times. Polybuffer was removed in the supernatant.

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TABLE I. Purification of DFT-Stimulating Substance

Step	Protein[a)] (mg/kg gland)	Activity[b)] (mg/kg gland)	Specific activity[c)] (mg/mg protein)	Recovery (%)
Isoelectric precipitation	14350	343	0.0239	100
Ultrafiltration	4972	317	0.0637	92.4
Sephadex G-75 (1st)	1235	232	0.188	67.6
Sephadex G-75 (2nd)	440	211	0.480	61.5
Chromatofocusing (1st)	107	106	0.991	30.9
Chromatofocusing (2nd)	37.0	37.0	1.000	10.8

a) Determined by the method of Lowry *et al.* *b*) Determined by the SRID method. *c*) Specific activity was defined as mg of DFT-stimulating substance per mg of protein.

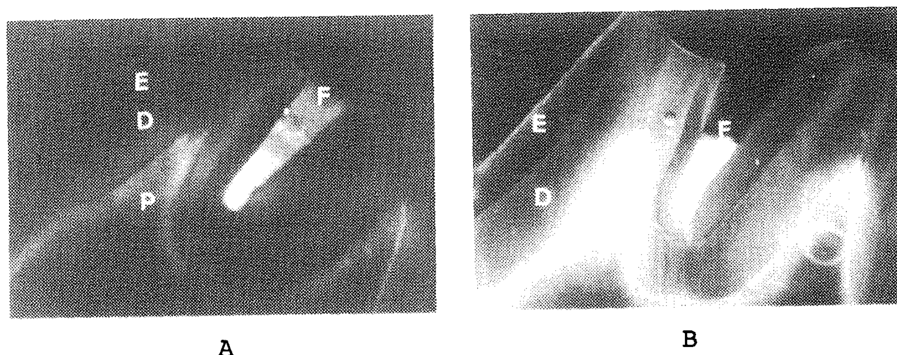

Fig. 3. Photomicrographs of Molar Sections from Rats on Bioassay

(A) Control: The molar section of a rat which was injected with physiological saline. The dentin was so opaque that no fluorescence could be observed in it.

(B) Positive sample: The molar section of a rat which was injected with 10 μg of the purified DFT-stimulating substance per kg. Fluorescence was observed in the dentin due to stimulation of fluid movement (transporting acriflavine hydrochloride), and the dentinal tubules showed brilliant green fluorescence.

P, pulp; D, dentin; E, enamel; F, food.

TABLE II. DFT-Stimulating Activity of the Fractions at Each Purification Step on Bioassay

Step	Dose (μg/kg)	Number of rats	Fluid movement[a)]
Ultrafiltration	100	7	3.29 ± 0.29[b)]
Sephadex G-75 (1st)	50	7	2.71 ± 0.29[c)]
Sephadex G-75 (2nd)	50	7	3.14 ± 0.34[b)]
Chromatofocusing (1st)	10	7	2.43 ± 0.20[c)]
Chromatofocusing (2nd)	10	7	3.00 ± 0.31[b)]
Control	—	7	1.14 ± 0.26

a) Mean ± standard error. *b*) $p < 0.01$, *c*) $p < 0.05$; *versus* control.

The precipitate was dissolved in 0.025 M imidazole–acetic acid buffer, pH 7.5, and the solution was dialyzed with several changes of the same buffer by ultrafiltration using a Diaflo YM5 membrane. Chromatofocusing was repeated in the same way on the 1st PBE 94 exchanger gel. The buffered solution was again applied to a column (2.5 × 50.9 cm) of PBE 94 exchanger gel. The eluted fractions 88—92 on rechromatofocusing (Fig. 2B) were also concentrated by the same method, desalted completely by ultrafiltration using a Diaflo YM5 membrane against distilled water, and lyophilized.

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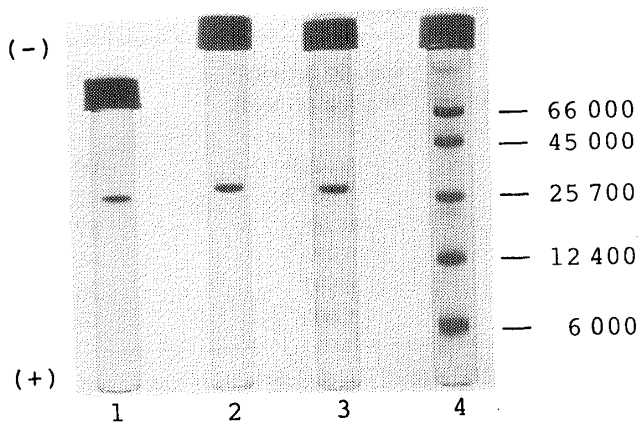

Fig. 4. Disc and SDS Polyacrylamide Gel Electrophoresis of Purified DFT-Stimulating Substance

1, disc gel; 2, SDS-gel in the absence of 2-mercaptoethanol; 3, SDS-gel in the presence of 2-mercaptoethanol; 4, SDS-gel of standard proteins.

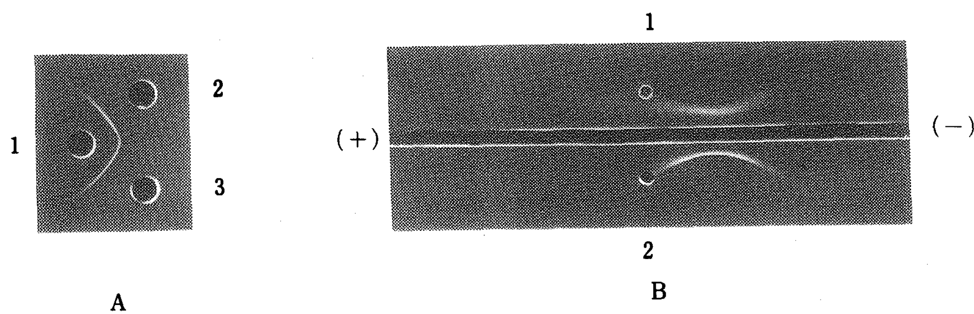

Fig. 5. Immunodiffusion and Immunoelectrophoresis of DFT-Stimulating Substance

(A) Immunodiffusion. Wells: (1) antiserum to purified DFT-stimulating substance, (2) ultrafiltrate, (3) purified DFT-stimulating substance.

(B) Immunoelectrophoresis. Trough: antiserum to purified DFT-stimulating substance. Wells: (1) ultrafiltrate, (2) purified DFT-stimulating substance.

A total of 37 mg of the purified DFT-stimulating substance was obtained from 1 kg of bovine parotid gland and the recovery as determined by the SRID method was 10.8% from the pH 5.0 supernatant. The specific activity of the purified preparation was increased 42-fold from the pH 5.0 supernatant. A dose of 10 μg of the purified preparation per kg of body weight was positive in the bioassay, as indicated by the fluorescence. A summary of the purification procedure is shown in Table I.

Typical photomicrographs of molar sections from rats used for biological assay are shown in Fig. 3. The molar sections were viewed through a fluorescence microscope to detect the movement of acriflavine. When fluid movement had occurred, the dentinal tubules fluoresced a brilliant green color (Fig. 3B). The fluid movement values of various fractions during the purification of the DFT-stimulating activity are shown in Table II.

Homogeneity of Purified DFT-Stimulating Substance

The purified preparation gave a single band that moved to the anode on 10% disc gel electrophoresis at pH 8.3, and appeared to be homogeneous. SDS-gel electrophoresis of the purified preparation gave a single band in the presence or absence of 2% 2-mercaptoethanol, suggesting that the substance consists of a single polypeptide chain (Fig. 4). Gel filtration of the purified preparation on a Sephadex G-75 column under the conditions used for estimation of the molecular weight gave a symmetrical peak. The purified preparation and crude ultrafiltrate each gave a single precipitin line against the antiserum to the purified preparation on double radial immunodiffusion analysis, and also gave a single precipitin arc with slight cathodal mobility on immunoelectrophoresis when cross-reacted with the antiserum to the purified preparation (Fig. 5).

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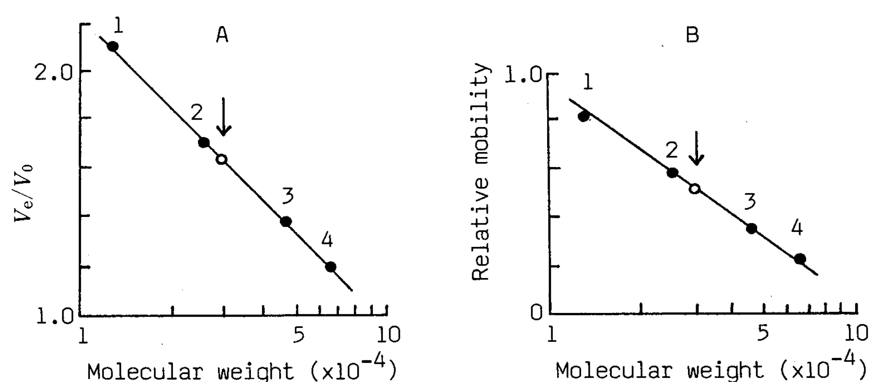

Fig. 6. Estimation of the Molecular Weight of DFT-Stimulating Substance

(A) Gel filtration on a Sephadex G-75 column.
(B) SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol.
The standard proteins used for calibration were: 1, cytochrome c; 2, chymotrypsinogen A; 3, ovalbumin; 4, bovine serum albumin. The positions of DFT-stimulating substance are indicated by arrows.

TABLE III. Amino Acid and Carbohydrate Compositions of DFT-Stimulating Substance

	Mol%[a]	Residues per molecule
Lys	5.8	15.4
His	3.5	9.2
Arg	3.6	9.5
Asp	12.6	33.2
Thr[b]	5.8	15.2
Ser[b]	6.7	17.7
Glu	9.3	24.4
Pro	8.8	23.3
Gly	7.5	19.8
Ala	6.0	15.7
Half-Cys[c]	0.1	0.2
Val[d]	7.3	19.2
Met	1.2	3.1
Ile[d]	1.9	4.9
Leu	10.3	27.1
Tyr	3.2	8.3
Phe	4.2	11.1
Trp[e]	2.2	5.8
Total		263.1
Hexose (%)	<0.1	
Hexosamine (%)	<0.1	
Sialic acid (%)	<0.1	

a) The amino acid contents were estimated as average or extrapolated values of 24—72 h hydrolysate. *b*) Value extrapolated to zero time. *c*) Determined as cysteic acid from an 18 h hydrolysate after performic acid oxidation. *d*) Value from 72 hydrolysate only. *e*) Determined by measuring the absorbance at 288 and 280 nm of the sample dissolved in 6 M guanidine hydrochloride.

Estimation of Molecular Weight

The molecular weight of the DFT-stimulating substance was estimated to be 30000 by gel filtration on a Sephadex G-75 column. The molecular weight of DFT-stimulating substance determined by SDS-gel electrophoresis was 30000 for the reduced sample, coinciding with the result of gel filtration, as shown in Fig. 6.

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Isoelectric Point

The isoelectric point of DFT-stimulating substance was estimated by isoelectric focusing to be pH 6.0.

Amino Acid and Carbohydrate Compositions

Table III shows the result of amino acid composition analysis after hydrolysis at 110 °C for 24 and 72 h. The numbers of residues per molecule of protein were calculated assuming a molecular weight of 30000. The DFT-stimulating substance consisted of a total of 263 amino acid residues per molecule. No half-cystine was detected. There was no detectable hexose, hexosamine or sialic acid (Table III).

Absorption Spectrum

The ultraviolet absorption spectrum of DFT-stimulating substance was measured in 0.02 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. An absorption maximum, λ_{max} was seen at 280 nm, and the extinction coefficient, $E_{1\,cm}^{1\%}$ at 280 nm was 15.4.

Discussion

Steinman *et al.*[6] have purified from porcine parotid glands a substance which stimulates fluid transport through the dentinal tubules in the molar teeth of rats. We found a similar active substance in bovine parotid glands, and this active substance was eventually purified by means of chromatofocusing. Steinman *et al.* used preparative electrophoresis as the final step in purification, but the amount of protein that can be purified by this technique is limited. In contrast, our technique of chromatofocusing can separate proteins according to their isoelectric points and is an excellent method by which to purify large quantities of proteins at one time while maintaining stable biological activity.

Using a bioassay, Steinman *et al.*[6] expressed fluid transport as fluid movement ratio (FMR), that is, the ratio of the number of occlusal grooves under which fluorescence was observed to the total number of occlusal grooves present. However, as occlusal grooves may exhibit autofluorescence depending on diet and microorganisms, it is not desirable to use only this parameter. Judgement depends on the strength of autofluorescence, and this can lead to error. Furthermore, because the molar sections were observed with a transmitted UV illumination-type fluorescence microscope, thin molar sections were necessary. In order to overcome these problems, we assessed fluid movement by observing whole molar sections, and we evaluated the strength of DFT-stimulating activity based on the whole images of the molar teeth to improve the reliability. Furthermore, as we used an incident illumination-type fluorescence microscope equipped with blue wavelength excitation, observation was easy even if the molar sections were thick.

The amount of DFT-stimulating substance in bovine parotid glands was approximately 400 mg per kg of gland. The content of active substance in porcine parotid glands[6] could not be measured by immunological quantitative analysis, nor was it clear from the results of bioassay, as the errors with this technique are large. Nevertheless, the content may be almost the same as that of bovine parotid glands, judging from the reported yields of the purified preparation.

Purified bovine DFT-stimulating substance was homogeneous on both disc polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis. The purified preparation also showed a single precipitin line on both immunodiffusion and immunoelectrophoresis. The molecular weight of this active substance was estimated to be 30000 by both Sephadex G-75 gel filtration and SDS-gel electrophoresis. The isoelectric point was found to be pH 6.0, and the active substance contained less than 1% carbohydrate. No half-cystine was detected, and there was no change in apparent molecular weight on SDS-gel electrophoresis in

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the presence of 2-mercaptoethanol, indicating that the molecule does not contain a disulfide bond. Thus, the active substance is considered to consist of a single polypeptide chain.

The bovine DFT-stimulating substance was found to have a molecular weight of 30000, a value which differs markedly from that of 8100 for the porcine substance (PH-A_β) reported by Steinman *et al.* Furthermore, bovine DFT-stimulating substance contained only 16.3% glycine and proline in total and had an isoelectric point of pH 6.0, whereas PH-A_β contained 73.3% glycine and proline in total and had an isoelectric point of pH 7.5. These differences in properties cannot be fully explained by the difference between animal species. Also we thought it unlikely that the active substance in bovine parotid glands would exist as a complex of a carrier protein and a polypeptide with a molecular weight of 8100 or as a precursor of the lower molecular weight form, judging from the amino acid composition. Thus, it seems likely that the substances purified from bovine and porcine parotid glands, although they have the same DFT-stimulating activity, are quite different chemical entities.

Although we attempted to isolate a substance corresponding to PH-A_β, no active substance with a molecular weight below 30000 was detected at any step of the purification. The minimum effective dose of the PH-A_δ fraction reported by Steinman *et al.*[6] was nearly equal to that of the bovine DFT-stimulating substance. However, the PH-A_δ fraction was a heterogeneous mixture and its amino acid composition was not reported. Therefore, it is not clear at present whether bovine DFT-stimulating substance is related to PH-A_δ.

In caries studies, investigations on proteins or peptides that suppress dental caries are obviously of great importance. An understanding of the mechanism of the suppression of dental caries by the DFT-stimulating substance is an important goal for future studies.

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