Isolation of a Mouse Submaxillary Gland Protein Accelerating Incisor Eruption and Eyelid Opening in the New-born Animal*

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During the course of our studies on the nerve growth-promoting protein of the submaxillary gland of the mouse (1, 2), it was noted that the daily injection of partially purified extracts of the salivary gland into new-born mice resulted in a number of gross anatomical changes in addition to the previously reported effects on the nerve cells. These were (a) precocious opening of the eyelids (as early as 7 days instead of the usual 12 to 14 days), (b) precocious eruption of the teeth (at 6 to 7 days instead of the normal 8 to 10 days), and (c) a marked stunting of the animals with an inhibition of hair growth.

We are reporting here the isolation of the factor responsible for the earlier development of the incisors and eyelids. The tooth-lid factor is a heat-stable, nondialysable, antigenic protein, whose most distinctive chemical characteristic is the absence of phenylalanine and lysine. The injection of 0.5 μg per 1.5 g of body weight per day of tooth-lid factor into new-born mice or rats produces a demonstrable biological effect.

EXPERIMENTAL PROCEDURE

Materials—The submaxillary glands were isolated from adult (over 24 g) male Swiss Webster mice. Carboxymethyl cellulose (CM-cellulose) and diethylaminoethyl cellulose (DEAE-cellulose) were the standard Selectacel reagents (Schleicher and Schuell Company). Sephadex G-25 and G-75 were obtained from Pharmacia Laboratories, Inc. Paper electrophoretic patterns were prepared with the instrument manufactured by the E-C Apparatus Company; immunoelectrophoretic runs were obtained with cellulose acetate strips and the Shandon apparatus (Consolidated Laboratories, Inc.).

The crystalline trypsin and chymotrypsin were obtained from the Worthington Biochemical Corporation. The crystalline bacterial proteinase (Novo) was a gift of Dr. B. Pogell.

Protein determinations were made either by the use of the Folin phenol reagent with bovine crystalline albumin as a standard (3), or by measuring the optical density at 280 μm. No differences were observed between the two types of diluent; no differences were observed between the two types of controls. The assay was sensitive to 2-fold dilutions. The material was injected with either distilled water or dilute salt solutions (less than 0.15 M) as diluent. Controls received either no injections or received diluent; no differences were observed between the two types of controls. The assay was sensitive to 2-fold dilutions of the active material (see Tables I and II).

PURIFICATION AND PROPERTIES

Effect of Crude Extracts—Crude 10% extracts of the submaxillary glands of adult male mice were prepared by adding 9 volumes of cold distilled water per g, wet weight, of tissue and homogenizing them in a Waring Blender or glass homogenizer for 3 minutes. The homogenate was then centrifuged at 16,000 × g for 10 minutes at 0-3°C, and the supernatant fluid was assayed by the daily subcutaneous injection into new-born mice. The results are shown in Table I. It can be seen that one injection of the 5% extract (a total of 0.3 mg of protein as measured with the phenol reagent) was lethal. The 2 and 1% extracts caused a marked growth inhibition, an inhibition of hair growth, and the precocious development of the incisors and eyelids. Control experiments were performed in which the effects of the injection of 10% extracts of a variety of mouse tissues were examined. These included pancreas, liver, kidney, adrenal gland, brain, pituitary, thymus, testes, and parotid gland. In no instance was any effect observed on the development of the eyelids or incisors. Most of the extracts showed a slight growth-inhibiting effect; the body weights at the end of 12 days were from 5 to 25% under the control values.

We have thus far been able to prepare biologically active extracts only from the submaxillary glands of male mice. Extracts (10%) of the submaxillary glands from adult female mice were not lethal and had no effects on development. Similarly, 10% extracts prepared from bull and cow submaxillary and parotid glands, male rabbit and guinea pig submaxillary glands, and sheep submaxillary glands were not lethal and had no discernible effects on the development of the eyes or teeth. Extracts (10%) of the submaxillary gland of the male rat had a
barely detectable effect, causing the eyelids to open 1 day earlier than those of the controls.

Purification—Adult male Swiss Webster mice weighing 24 g or more were killed with chloroform. The submaxillary glands were excised and stored frozen until a sufficient quantity had been accumulated. Approximately 22 g, wet weight, of glands were obtained from 150 mice. The tissue may be stored frozen for at least several weeks with no detectable loss in activity. For each 22 g of frozen tissue were added 90 ml of cold distilled water, and the mixture was homogenized in a Waring Blender for 3 minutes. The homogenate was centrifuged for 10 minutes. All centrifugations in this procedure were performed at 0-3°C and 16,000 X g in a Servall centrifuge. The supernatant fluid was decanted and the residue was stirred with 80 ml of cold distilled water and recentrifuged. The supernatant fluids, containing 4.2 g of protein (Folin phenol reagent, bovine albumin as a standard) were combined, and the residue was discarded. The daily injection of 120 μg of protein per 1.5 g of body weight resulted in the opening of the eyelids at 9 days.

To 9 volumes of extract was added 1 volume of a stock streptomycin solution made of 1.46 g of streptomycin sulfate adjusted to a final volume of 20 ml and containing sufficient 1 M NaOH to make the final pH approximately 9.0. The final pH of the mixture was between 6.8 and 7.1. The mixture was allowed to stand at 0°C overnight and then was centrifuged for 5 minutes. The clear red supernatant fluid was retained, and the residue (e.g., nucleoproteins) was discarded. To the supernatant liquid (178 ml) were added 100 g of solid ammonium sulfate, and the mixture was allowed to stand at 0°C for 30 minutes. The precipitate was separated by centrifugation for 10 minutes, and the supernatant was discarded. The precipitate was suspended in 15 ml of water and dialyzed with stirring for 24 hours against five changes of 2-liter amounts of distilled water. The final volume was 112 ml, containing 2.3 g of protein. The daily injection of 75 μg of protein per 1.5 g of body weight resulted in the opening of the eyelids at 9 days.

The solution was transferred to a 250-ml flask and placed in a boiling water bath for 5 minutes. After cooling, the mixture was centrifuged for 10 minutes. The supernatant fluid was decanted, and the residue was washed with 30 ml of water and recentrifuged. The supernatant fluids were combined, and the solution was then dialyzed overnight against distilled water. The dialyzed solution contained 356 mg of protein and 257 O.D. units. (An O.D. unit was defined as the optical density at 280 μm in a 1-cm cell multiplied by the number of milliliters in the sample. The O.D. unit as a measure of the amount of protein was employed in the subsequent chromatographic procedures.) A second column of CM-cellulose was prepared, and 3 g of the powder in a column 1.5 cm in diameter was washed successively with the following solutions: (a) 0.5 M sodium hydroxide and 0.5 M sodium chloride, (b) water, until alkali-free. (c) 0.1 M sodium acetate buffer, pH 5.6, and (d) 0.002 M sodium acetate buffer, pH 5.6. The dialyzed solution described above was then passed through the column at a flow rate of 0.2 to 0.3 ml per minute. These and all subsequent chromatographic procedures were carried out at room temperature. The column was then washed with 40 ml of distilled water. Under these conditions, the active material was not adsorbed. The combined filtrates Fraction CM-1 contained 161 O.D. units. Most of the protein remaining on the column could be removed with 1 M sodium chloride and was biologically inactive.

A column of CM-cellulose (54 O.D. units) and 80% in Fraction CM-2b (48 O.D. units). The daily injection of 0.004 μg of protein (0.014 O.D. unit) per 1.5 g of body weight caused the opening of the eyelids in 9 days. Boiling the solution for 30 additional minutes did not alter the biological activity.

A column of CM-cellulose was prepared as follows: 1 g of the powder in a column 1.5 cm in diameter was washed in a manner identical with that described above, except that the pH of the acetate buffer was 4.2. The CM-1 fraction was then adjusted to pH 4.2 with 1 M acetic acid (approximately 0.8 ml), passed through the column, and washed with 60 ml of distilled water. The combined filtrates contained 50 O.D. units. The active material was then eluted with a sodium chloride gradient prepared by allowing 0.2 M NaCl to flow into a 140-ml constant volume mixing chamber containing distilled water. Approximately 2.6-ml fractions were collected, and their optical densities at 280 μm were determined. The results are shown in Fig. 1A.

Since the available bioassay could only detect, at best, 2-fold changes in specific activity, broad fractions under discernible peaks were combined and assayed. Thus, the unadsorbed portion and Fractions CM-2a (tubes 13 to 38, Fig. 1A) and CM-2b (tubes 39 to 60, Fig. 1A) were compared. The unadsorbed eluate (50 O.D. units) contained only traces of activity; approximately 20% of the total activity was detected in Fraction CM-2a (54 O.D. units) and 80% in Fraction CM-2b (48 O.D. units). The daily injection of 0.004 O.D. unit (Fraction CM-2b) per 1.5 g of body weight resulted in the opening of the eyelids at 9 days. The further purification was made with Fraction CM-2b.

Fraction CM-2b was dialyzed with stirring against distilled water for 6 hours and then lyophilized to approximately 1 ml. After thawing, the turbid solution was clarified by the addition of a few milligrams of solid NaHCO₃. The clear solution was then fractionated on a column of Sephadex G-75, 1.5 x 112 cm; 2.5-ml fractions were collected, with 0.004 M NaCl as eluent, and the optical density at 280 μm was determined. The results are plotted in Fig. 1B. Significant amounts of biological activity could be detected only under the major peak between tubes 53 and 63. Yield was 33 O.D. units. Tubes 25 to 52 contained 5 to 10% of the applied activity and were not further examined.

**TABLE I**

<table>
<thead>
<tr>
<th>Concentration of extract*</th>
<th>Animal No.</th>
<th>Eyelids open</th>
<th>Incisors erupt</th>
<th>Weight on day 10</th>
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<td>%</td>
<td></td>
<td>day</td>
<td>day</td>
<td>e</td>
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<td>1</td>
<td>1</td>
<td>8</td>
<td>6</td>
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</tr>
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<tr>
<td></td>
<td>6</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
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</tr>
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<td>13</td>
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<td>6.1</td>
</tr>
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*The injection of 5% extracts was lethal within 24 hours.
A column of DEAE-cellulose was prepared, and 2 g of the powder in a column 1.5 cm in diameter were washed in a manner identical with that described for the CM-cellulose column, with the acetate buffer, pH 5.6. The Sephadex fraction (tubes 53 to 63) was passed through the column and washed with 30 ml of water. The filtrate contained only traces of protein (less than 0.5 O.D. unit) and no detectable activity. The active material was then eluted with a NaCl gradient prepared by allowing 0.2 M NaCl to flow into a 140-ml constant volume mixing chamber containing distilled water. Fractions of 2.5 ml were collected. The results are shown in Fig. 1C. Activity could only be detected under the major peak. Yield was 19 O.D. units (tubes 48 to 64).

A column of CM-cellulose was prepared with 1 g of the powder in a column 0.9 cm in diameter. The column was equilibrated as described, with the acetate buffer, pH 4.2. The DEAE-cellulose fraction was concentrated by lyophilization to approximately 2 ml and desalted by passing it through a column of Sephadex G-25, 1.1 × 48 cm. The pH was adjusted to 4.2 with 0.1 M acetic acid, and the solution was passed through the column, which was subsequently washed with 30 ml of 0.002 M acetate buffer, pH 4.2. The combined filtrates contained 1.2 O.D. units and were discarded. The active material was then eluted with a pH gradient prepared by allowing 0.01 M sodium acetate to flow into a 60-ml constant volume mixing chamber containing 0.01 M sodium acetate buffer, pH 4.2. Fractions of 3 ml were collected, and the results of the optical density measurements are shown in Fig. 2. After the tubes under each peak were pooled (tubes 31 to 39 and 40 to 54), both fractions, within the limits of the biological assay, had the same specific activity. The yields were 12 O.D. units in tubes 40 to 54 and 3 O.D. units in tubes 31 to 39. In five preparations of tooth-lid factor, the final total yields ranged from 12 to 17 O.D. units (4.6 to 6.6 mg); the ratio of the amounts of material in the two peaks was quite variable, ranging from 4:1, as in this preparation, to over 30:1.

After tubes 40 to 54 were pooled, the injection of 0.002 O.D. unit (0.8 µg) per 1.5 g of body weight resulted in the lid opening at 9 days. Thus, approximately 20% of the original material was recovered with a purification of about 150-fold, based on protein content.

Individual assays of the tubes under the major peak shown in Fig. 2 at the standard dosage of 0.025 ml per 1.5 g of body weight gave the following results: tube 42 (O.D., 0.180), lids opened on day 8; tube 43 (O.D., 0.340), lids opened on day 7; tube 48 (O.D., 0.375), lids opened on day 7; and tube 51 (O.D., 0.170), lids opened on day 8. Thus, within the limits of the assay, the activity was uniformly distributed under the eluted peak.

All subsequent experiments were performed with the pooled
TABLE II

Effect of injection of purified tooth-lid factor into new-born mice

New-born mice were treated with daily injections of 0.025 ml per 1.5 g of body weight. Controls received no injections. The effects on three litters, A, B, and C, are recorded.

<table>
<thead>
<tr>
<th>Dosage (µg/1.5 g/day)</th>
<th>Animal No.</th>
<th>Eyelid open day</th>
<th>Incisors erupt day</th>
<th>Weight on day 10</th>
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The material was stained with a solution of 0.4 g of bromophenol blue dissolved in 1 liter of 95% alcohol saturated with mercuric chloride. The results are shown in Fig. 5. Only a trace of color was detectable with a ninhydrin spray reagent. From companion strips, the protein band could be eluted, and, upon injection into new-born mice, the lid opening and tooth eruption effects were elicited.

The compound was then subjected to paper electrophoresis for 16 hours with a variety of buffers, and 50-µg quantities were applied as a spot to the centers of strips of Whatman 3 MM paper. Glucose was used as an indicator of electroosmosis; the protein stain was used to detect the tooth-lid factor. The results are shown in Fig. 6. Again, only a single major component was detectable, with some tailing in the more acidic buffers. From a companion strip at pH 6.5, the protein band was eluted and found to be biologically active. From the relative position of the glucose (Fig. 6), the apparent corrected mobilities were calculated, and the results indicated that the factor had an isoelectric point at pH 4.2.

The tooth-lid factor is antigenic. The preparation and properties of the antiserum are described in a later section. Upon examination of the factor by electrophoresis and immunoelectrophoresis with cellulose acetate strips, again only one electrophoretic and only one precipitating band could be detected. These results are shown in Fig. 7.

The biological activity of the material is destroyed upon incubation with crystalline chymotrypsin or bacterial proteinase.
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FIG. 4. Ultracentrifugation pattern of the tooth-lid factor at a concentration of 4 mg per ml in 0.15 M NaCl, 0.1 M Tris, and 0.001 M Versene, adjusted to pH 7.86. The pictures were recorded at 8-minute intervals at 59,780 r.p.m. in a valve-type synthetic boundary cell.

(Novo), and it is partially inactivated by trypsin. In these experiments, 300 μg of the factor were incubated with 100 μg of the proteolytic enzyme in 0.6 ml of 0.035 M sodium phosphate buffer, pH 7.1, for a period of 9 hours at 37°. In control incubations, the mixtures were boiled for 2 minutes immediately after the addition of the enzyme. After the incubation was completed, the tubes were heated in a boiling water bath for 2 minutes, diluted to 2 ml with water, and assayed. The results were as follows: boiled enzyme controls, lids opened on day 7; bacterial proteinase incubation, lids opened on day 12; chymotrypsin incubation, lids opened on day 11; trypsin incubation, lids opened on day 9; and uninjected controls, lids opened at day 12. Gross examination of the mice indicated that the destruction of the lid opening effects was accompanied by the destruction of the tooth erupting effects.

The biological activity of the tooth-lid factor is stable to boiling in distilled water for 30 minutes but is destroyed upon heating in 0.1 N NaOH for 1 hour or 0.2 N HCl for 2 hours in a boiling water bath.

Amino Acid Composition—Of the purified protein, 600 μg were hydrolyzed for 22 hours in a vacuum in a sealed tube with 3 ml of constant boiling HCl at 110°. After removal of the HCl in a vacuum desiccator over solid NaOH and silica gel, 200-μg aliquots were chromatographed two-dimensionally on Whatman No. 1 paper with the following solvent pairs: first direction, n-propanol-water-ammonia (6:3:1); and in the second direction, the upper phase of a mixture of tert-amyl alcohol-water-formic acid (3:3:1). The completed chromatograms were sprayed with 0.1% ninhydrin in butanol. The results are shown in Fig. 8. From the relative Rf values it was determined that all of the common amino acids were present except lysine and phenylalanine. No unidentifiable ninhydrin-positive material was detected. In a separate chromatogram, after the protein was hydrolyzed as described above for 18 hours at 106°, no detectable reducing sugar was found with use of a 300-μg aliquot of the protein and spraying the paper with aniline hydrogen phthalate (5). By this procedure, 5 μg of glucose were detectable.

The absence of lysine and phenylalanine was confirmed by direct analysis of a separate preparation of the material with a Beckman automatic amino acid analyzer. Samples of 0.9 to 1.1 mg of the protein were hydrolyzed in a vacuum with 3 ml of constant boiling HCl and heating for 22 hours at 110°. The results of the analysis are shown in Table III. The minimal molecular weight, as determined from the amino acid ratios with the assumption of 1 alanine residue per molecule, was 14,038. The amino acids and ammonia recovered accounted for 85% of the weight of the sample.

The material was examined by the procedure of Boyer (7) for the detection of sulfhydryl groups. A 1.5 X 10⁻⁵ M solution of the factor (assuming a molecular weight of 15,000) showed a sulfhydryl content of less than 0.2 X 10⁻⁵ μ, indicating the absence of cysteine.

FIG. 5. Ascending paper chromatography of the tooth-lid factor in A, propanol-water-ammonia, and B, butanol-water-acetic acid. The line indicates the starting position, and the arrow, the solvent front.
**Preparation of Antiserum.**—Each of two rabbits received injections of 2 mg of Fraction CM-2b in the footpads with the Difco preparation of Freund's complete adjuvant (0.5 ml of an aqueous solution of the antigen emulsified with 0.5 ml of the adjuvant). After 6 weeks the injection was repeated, again with the adjuvant. The animals were then bled from the ear vein 4 weeks after the second injection.

The assay for antibody titer was based on the inhibition of the biological activity of the antigen when injected into the newborn mouse. The most purified preparation (100 μg) in 0.5 ml of isotonic sodium chloride was incubated with 1.5 ml of a series of 2-fold dilutions of the rabbit serum. The mixture was incubated for 1 hour at 24°C and then for 2 hours in a refrigerator. The precipitates which formed were then centrifuged, and 0.025 ml of the supernatant liquid was injected per 1.5 g of body weight of new-born mice. In control experiments, in which isotonic sodium chloride or normal rabbit serum was used, no precipitation was observed, and upon bioassay, these preparations resulted in the opening of the eyelids at 8 days. In the experimental run, the supernatant fluid from tube 1 (containing undiluted serum) was used to prepare buffers of pH 3.8, 4.3, and 4.8; KH₂PO₄-Na₂HPO₄ was used for pH 6.5; and barbital-sodium barbital, for pH 8.7. The electroosmotic flow is indicated by the line representing the glucoside spot, visualized in parallel strips by spraying with aniline hydrogen phthalate.

**Fig. 6.** Paper electrophoresis of the tooth-lid factor. The specimens were applied at the center line. All of the runs were for 16 hours at a field strength of 9 volts per cm in buffers of 0.1 ionic strength, except for the barbital buffer, which had an ionic strength of 0.07. Sodium acetate-acetic acid mixtures were used to prepare buffers of pH 3.8, 4.3, and 4.8; KH₂PO₄-Na₂HPO₄ was used for pH 6.5; and barbital-sodium barbital, for pH 8.7. The electroosmotic flow is indicated by the line representing the glucose spot, visualized in parallel strips by spraying with aniline hydrogen phthalate.

**Fig. 7.** Electrophoretic (A) and immunoelectrophoretic (B) patterns of the tooth-lid factor. Cellulose acetate strips (16 X 2.5 cm) were used, with 0.07 ionic strength sodium barbital buffer, pH 8.6. The sample (20 μg of protein) was applied approximately one-fourth the distance from the anode end, as indicated by the arrow. The run was performed at room temperature for 3.5 hours at 0.5 ma per cm of width of the strip. In the immunoelectrophoretic run, the diffusion under oil was allowed to proceed for 20 hours. Ponceau S was used to stain the protein. All of the procedures and apparatus used are described by Kohn (4).
cubation with chymotrypsin or bacterial proteinase. The factor is antigenic when injected into rabbits, and the antiserum thus obtained will precipitate the biologically active antigen. The biological activity is associated with the single protein band obtained by paper electrophoresis or paper chromatography. The ultraviolet absorption spectrum and amino acid content of the material are consistent with this view.

Heat stability and the absence of lysine and phenylalanine are the most striking of its chemical characteristics. This absence may also be considered as a criterion of purity, since it might be expected that contaminating polypeptides would contain these amino acids.

The nature of the minor active peak in Fig. 2 has not been ascertained. The possibility exists that the relatively small amounts of activity present in the fractions discarded during the purification may also differ from the tooth-lid factor as finally isolated.

The mechanism by which tooth-lid factor elicits such diverse effects as incisor eruption and eyelid opening is not known. A

TABLE III
Amino acid composition of tooth-lid factor

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<tr>
<th>Amino acid</th>
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Total (ammonia omitted) 121
Estimated molecular weight 14,638

* In a second run with a different preparation of the material and hydrolysis for 18 hours at 105°, again no phenylalanine and only a trace of lysine were detectable. The nearest whole numbers of amino acid residues per molecule were identical with those shown above, except for valine, isoleucine, serine, tyrosine, and aspartic acid, for which values of 4, 15, 12, and 20, respectively, were obtained.
histological study of the effects is in progress and may shed some
light on the process. It is not clear whether the factor acts
directly on the tissues involved or by way of some other systemic
(hormonal?) process.

It has been reported (8, 9) that thyroxine, when injected into
new-born rats, causes a precocious eruption of teeth and opening
of the eyelids. Daily doses just under the lethal level resulted
in lid separation at 11\(\frac{1}{2}\) days of age. At a dosage level of 1 \(\mu g\)
per 1.5 g of body weight, the tooth-lid factor will elicit lid separa-
tion in the new-born rat at 6 to 7 days of age. In addition, the
complete inactivation of the isolated protein by boiling in 0.1 N
NaOH (thyroxine is stable under these conditions) and the
inactivation with proteolytic enzymes indicate that our prepara-
tion was not contaminated with thyroxine. Very large doses of
cortisone (100 \(\mu g\) or more per day) have also been reported to
effect lid separation and incisor eruption in rats (10-12). Here
again, lid separation was advanced only 2 to 3 days, as against
the 7 to 8 days obtained with the tooth-lid factor (the lids of the
new-born rat normally open at 14 days). The possibility exists
that the factor isolated in the present paper is involved in these
hormonal effects.

In view of the reports that parotin, a protein isolated from
salivary glands, affects various aspects of calcium metabolism
(13, 14), we have injected parotin (Lot No. 90-B, Teikoku
Hormone Manufacturing Company) into new-born mice. With
daily doses of up to 100 \(\mu g\) per 1.5 g of body weight, no detectable
effect on lid opening or incisor eruption was observed.

The tooth-lid factor has thus far been tested only in new-born
mice and rats and is effective in both species. No observable
effects were noted upon the daily injection of the rabbit anti-
serum into either new-born mice or rats. The questions of
whether the factor is actually synthesized in the submaxillary
and eyelid development.

SUMMARY

1. A heat-stable protein has been isolated from the male
mouse submaxillary gland which, upon injection, elicits pre-
ocious tooth eruption and eyelid separation in the new-born mouse
and rat.

2. The preparation appears homogeneous when examined by
paper electrophoresis, paper chromatography, and immuno-
electrophoresis. Only one component with a sedimentation
constant of 1.25 S may be detected in the ultracentrifuge.
The protein has an isoelectric point at pH 4.2.

3. The amino acid analysis of the protein shows that lysine
and phenylalanine are absent. All of the other common amino
acids are present. The minimal molecular weight, estimated
from the assumed number of amino acid residues, is approxi-
mately 15,000.

4. The biological activity of the isolated tooth-lid factor is
destroyed by incubation with chymotrypsin or a crystalline bac-
terial proteinase, and it is partially inactivated by trypsin.
The factor is antigenic and may be precipitated with the aid of a
specific rabbit antiserum.

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Stanley Cohen


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