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CHEMICAL CHARACTERIZATION OF THE TWO FORMS OF EPIDERMAL GROWTH FACTOR IN MURINE SALIVA

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Extracts of murine salivary glands contain two molecular forms of epidermal growth factor, EGF I and EGF II (Petrides, P.E., Levine, A.E., Shooter, E.M.in: Peptides:-Synthesis, Structure and Function (Rich, D.H., Gross, E.eds.)p.781 (1981)). We have identified both molecules not only in salivary gland extracts but also in saliva using only reverse phase liquid chromatography methodology. EGF I and II were isolated from submaxillary gland extracts in a ratio of 3:1 regardless of whether the classical isolation procedure or our rapid RPLC based technique was used. Both molecular forms were present in the same ratio in saliva of mice of both sexes when salivation was induced by epinephrine treatment of the animals. As judged by amino acid analysis and N-terminal sequencing salivary EGF I corresponds to the 53 amino acid sequence of murine EGF and EGF II is Des-ASN-EGF. The observation that EGF and Des-ASN-EGF are cosecreted into saliva upon epinephrine stimulation implies a physiological role of EGF II which may be related to the high molecular weight EGF-complex.

The salivary gland contains several polypeptides which exert regulatory influences upon the proliferation and differentiation of various target cells (1). Among the factors that have been

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Abbreviations: RPLC, reverse phase liquid chromatography; EGF, epidermal growth factor; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; RIA, radioimmunoassay

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purified and characterized from this tissue is epidermal growth factor which stimulates the proliferation of the basal layer of various epithelia in vivo (2). EGF is secreted into saliva upon adrenergic stimulation, indicating that the gland serves as an exocrine organ for the production and secretion of the polypeptide (3). Because the glandular EGF content is androgendependent, much higher amounts of EGF are found in the glands of male mice than in those of female mice as determined by radioimmunological methods (4). Saliva EGF stimulates the proliferation of organotypic cultures of cornea suggesting the molecule is secreted into saliva in a biologically active form (5). EGF present in saliva may be important for wound healing in rodents, because the removal of the salivary glands causes deceleration of wound contraction (6). Moreover, topical application of EGF to wounds in sialectomized animals or communal licking of sialectomized by sham operated animals enhances closure of the wounds (7).

EGF was originally purified from the submaxillary gland by a combination of ion exchange chromatography and gel filtration (8). In 1972, a new procedure (9) was devised which made use of the observation that EGF selectively adsorbs to polyacrylamide gels at low pH (1.5) and is therefore strongly retarded in gel filtration on such columns. The procedure in combination with anion exchange chromatography yielded homogeneous EGF allowing the determination of the sequence of the molecule (10). When partially purified EGF preparations are subjected to reverse phase liquid chromatography (RPLC), two molecular forms of EGF (EGF I and EGF II) are seen (11-14). It is conceivable that one of these EGF forms represents an immuno- and bioactive degradation product which has been formed during tissue extraction or during purification e.g. as a consequence of exposure to low pH for prolonged time during chromatography according to the standard procedure (9). We have therefore determined the chemical nature of the two forms and investigated whether one of them possibly represents an artefactual degradation product from the isolation procedure.

We report here the isolation of EGF I and EGF II from saliva and submaxillary gland extracts using only reverse phase liquid chromatography and the structural characterization of these two forms. Because male and female glands differ in their immunoreactive EGF content, we also have isolated both forms from

female saliva and demonstrate that they are identical to the respective male forms.

MATERIALS AND METHODS

Materials - 60 days old male and female Swiss-Webster mice (Simonson, CA) were kept under controlled temperature and lighting conditions for seven days before collection of saliva or sacrifice. For the preparation of saliva the mice were anaesthesized by intraperitoneal injection of 60 µg pentobarbital (Nembutal®, Abbott Laboratories, Chicago, Ill.)/g body weight. After 10 minutes the animals were intraperitoneally injected with epinephrine (Park Davis, Morris Plains, N.J.) (4 µg/g body weight), the saliva collected with capillary tubes and immediately frozen on dry ice. For the preparation of tissue homogenate supernatant the animals were killed through carbon dioxide anaesthesia, the submaxillary glands immediately removed, rinsed in 0.2 M acetic acid and frozen in liquid nitrogen.

Extraction procedures - Saliva was either mixed with an equal volume of 0.36 M pyridine formate (pH 3.0) and centrifuged at 7500 x g for 5 min to remove particulate matter or acidified with HCl to pH 1.0, incubated for 60 min at 4°C and subsequently centrifuged. Prior to chromatography the solutions were readjusted to pH 3.0 with 0.36 M pyridine formate (pH 3.0). Submaxillary glands were extracted by homogenization (in a commercial Waring blendor) in 0.05 M acetic acid (8 ml/g frozen tissue) for 180 seconds; the homogenate was centrifuged for 30 minutes at 100.000 x g, the resulting pellet rehomogenized in 0.05 M acetic acid and centrifuged for 30 minutes. The supernatants of both centrifugations were pooled, filtered through nylon gauze and lyophilized. The lyophilizate was redissolved in 10 ml HCl (1N) and adjusted with pyridine formate to pH 3.0. Alternatively, the supernatant was not lyophilized and subjected to RPLC either directly or after adjustment with HCl to pH 1.0, incubation at 4°C for 60 min and readjusting to pH 3.0.

Chromatographic procedures - The RPLC system has been described (15). Reverse phase columns used were: RP8 (2.5 x 50 cm, particle size 10 µm, pore size 100 A, Whatman, Clifton, N.J.), Aquapore RP 300 (0.7 or 0.46 cm x 25 cm, particle size 10 µm, pore size 300 A, Brownlee Laboratories, Santa Clara, CA). Column eluates were monitored with a fluorometric postcolumn derivatization system with fluorescamine (16) and/or by UVabsorbance at 280 nm. Mobile phases utilized were 0.36 M pyridine formate (pH 3.0)/n-propanol (17), 0.25 M triethylammonium phosphate (pH 3.0)/acetonitrile (18), 0.1 % trifluoroacetic acid (TFA)(pH 2.10)/acetonitrile or 0.05% heptafluorobutyric acid (HFBA)(pH 2.48) with acetonitrile (19).

Structural analysis - For amino acid analysis a Liquimat III amino acid analyzer (Kontron, Zurich, Switzerland) equipped with an o-phthaldehyde fluorescence detection system (20) and a proline conversion accessory (21) was used. Peptide aliquots of 10 to 20 pmol were subjected to acid hydrolysis and analyzed using micromethodology (22). For amino acid sequence analysis pmol amounts of the purified samples were applied either to a modified spinnning cup sequenator or a City of Hope built gas phase sequenator (23-25). Phenylhydantoin (PTH) amino acid derivatives were analysed by RPLC. The PTH-derivatives of cysteine which are not stable (multiple breakdown products are observed) were not positively identified in this study.

Radioimmunological assay (RIA) - Plastic 96 well microtiter plates (Dynatech. Laboratories, Alexandria, Va.) were exposed to a solution of protein A (0.1 mg/ml; Pharmacia, Uppsala) for two hours at room temperature. The microtiter wells were then rinsed three times with phosphate buffered saline (PBS) (pH 7.4) containg 2mg/ml bovine serum albumin (BSA) and subsequently coated with a 1:100 dilution of a rabbit anti-mEGF serum (Collaborative Research, Lexington., Mass.). After 120 min the wells were washed with PBS/BSA solution. A mixture of ^{125}I-labelled EGF I (26) and either a known quantity of EGF I or a sample of unknown concentration were added. At the end of the incubation period of 120 min the unbound radiolabeled ligand was removed by washing with PBS/BSA solution; the wells were then cut out and the radioactivity counted. A standard curve for EGF was established with triplicate determinations in the concentration range of 20 pg to 20 ng/well.

RESULTS

Isolation of EGF I and II from male submaxillary glands- Preparative RPLC of lyophilized tissue extract revealed one major radioimmunoactive peak eluting at 25.2 % n-propanol (Fig. 1). In addition, a minor immunoreactive peak was observed at 20.4% n-propanol. Rechromatography of the four fractions representing the major immunoreactive peak on a semipreparative RP 300 column in pyridine formate/n-propanol showed one immunoreactive peak and several other polypeptides without immunoreactivity (Fig. 2). Further purification of the immunoreactive material on an analytical reverse phase column resulted in the separation of two immunoreactive peaks (Fig. 3). Monitoring the eluting polypeptides by both UV-absorption at 280 nm and subsequent fluorometric detection showed differences in UV-absorption and fluorometric yields for both molecules (Fig. 3).

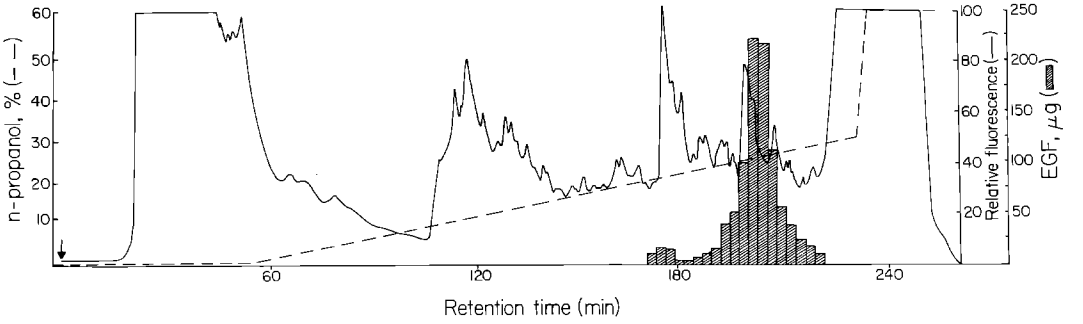
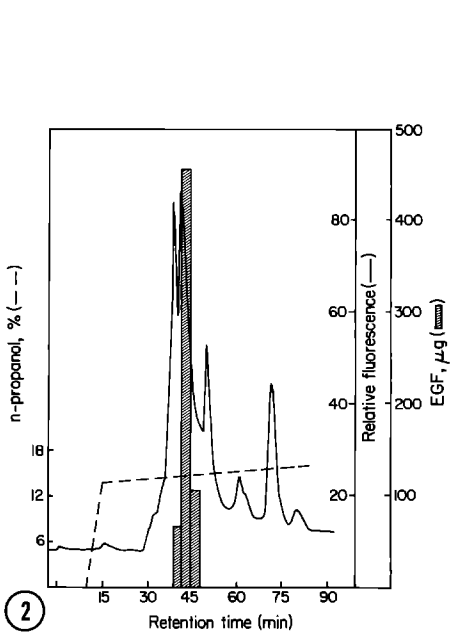


FIG. 1. **Preparative RPLC of submaxillary gland extract.** Submaxillary gland extract (originating from 25 glands (7.5g tissue)) was pumped on a preparative RP 8 column using the pyridine formate buffer with a gradient of n-propanol for elution. Flow rate 2.5 ml/min. 10 ml fractions were collected and 0.5 μl aliquots used for RIA.



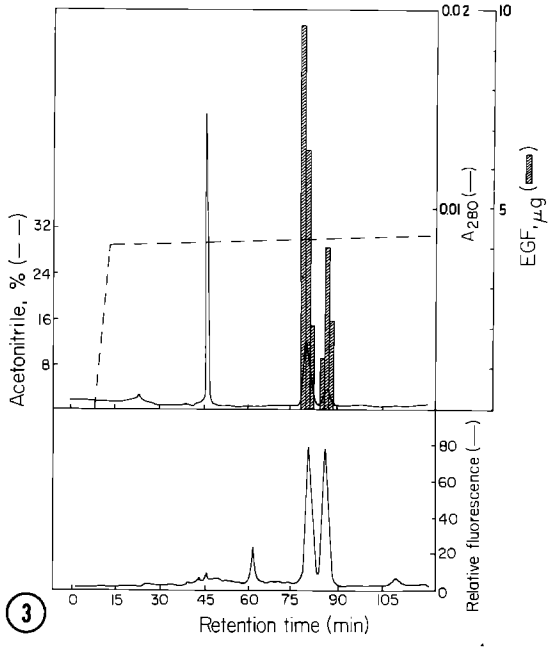


FIG. 2. **Rechromatography of immunoreactive material from preparative RPLC.** Fractions 73 - 77 (Fig. 1) were loaded after 1:1 dilution with pyridine formate on a semipreparative RP 300 column in pyridine formate (pH 3.0). The column was eluted with a linear gradient from 14 to 15 % n-propanol over 75 min. Flow rate 1.0 ml/min. 3 ml fractions were collected and 0.5 μl aliquots used for the RIA.

FIG. 3. **Final purification of EGF I and II.** Rechromatography of the major immunoreactive fractions from the semipreparative RPLC (Fig. 2) on an analytical RP 300 column in 0.1% TFA/CH_3CN. The column was eluted with a linear gradient from 28.8 to 30.4 % acetonitrile over 120 min. Flow rate 0.5 ml/min. 0.5 ml fractions were collected and 0.5 μl aliquots used for RIA. Polypeptides were monitored by UV-detection at 280 nm (upper panel) and postcolumn derivatization with fluorescamine (lower panel) with the detectors arranged in series at the column exit.

Isolation of EGF I and EGF II from male and female saliva- Semipreparative RPLC of male saliva revealed the presence of two EGF species (Fig. 4). Rechromatography of both peaks on an analytical RP 300 column in TFA/acetonitrile indicated that the two forms corresponded to EGF I/II of submaxillary gland extract (as judged by retention times) and appeared to be homogeneous (Fig. 5). Chromatography of female saliva under the same conditions yielded identical results except that EGF quantities found were approximately ten times lower (not shown).

Influence of acid treatment of tissue extracts and saliva prior to RPLC upon the yield of immunoreactive EGF- When the tissue extract supernatant was adjusted with pyridine formate to

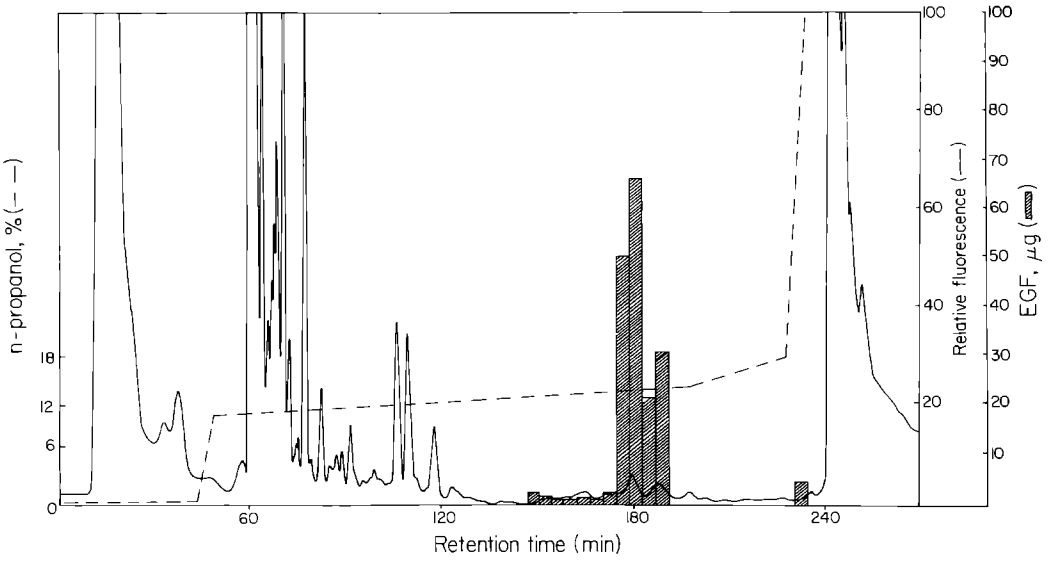


FIG. 4. **RPLC of male saliva.** 200 ul male saliva was mixed with an equal volume of pyridine formate (pH 3.0) and subjected to RPLC on a semipreparative RP 300 column using pyridine formate/n-propanol as mobile phase. Polypeptides were eluted with a linear gradient from 10.8 to 14.4 % n-propanol over 150 min. Flow rate 1.0 ml/min. 3.5 ml fractions were collected and 10 μl aliquots used for RIA.

pH 3.0 and then applied to the preparative RPLC system, a chromatogram different from that shown in Fig. 1 was observed (not shown). In addition, the yield of immunoreactive EGF dropped

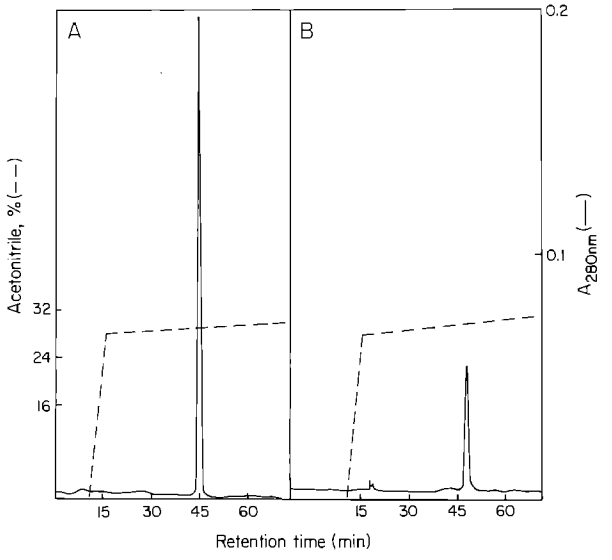


FIG. 5. **Final purification of EGF I and II.** Rechromatography of EGF I (panel A) and EGF II(panel B) on an analytical RP 300 column in TFA/CH_3CN. Flow rate 0.5 ml/min. 1 ml fractions were collected and 5 μl aliquots used for RIA.

Table I. **Amino acid composition[a] of murine EGF I and EGF II from male and female saliva**

	Male Saliva		Female Saliva		EGF[b]
	EGF I	EGF II	EGF I	EGF II	
ASX	6.63 ± 0.10	5.83 ± 0.01	6.64 ± 0.31	5.64 ± 0.20	7
THR	1.62 ± 0.01	1.80 ± 0.06	1.70 ± 0.09	1.69 ± 0.09	2
SER	5.78 ± 0.01	5.80 ± 0.20	5.81 ± 0.29	5.93 ± 0.29	6
GLX	3.50 ± 0.01	4.05 ± 0.25	3.32 ± 0.21	3.67 ± 0.38	3
GLY	6.36 ± 0.06	5.76 ± 0.10	6.61 ± 0.51	7.14 ± 0.28	6
ALA	0	0	0	0	0
VAL	1.46 ± 0.02	1.75 ± 0.25	1.68 ± 0.07	1.68 ± 0.14	2
MET	1.26 ± 0.02	1.18 ± 0.05	0.93 ± 0.13	0.86 ± 0.03	1
ILE	1.53 ± 0.02	1.54 ± 0.02	1.89 ± 0.43	1.68 ± 0.22	2
LEU	4.57 ± 0.02	4.55 ± 0.01	4.51 ± 0.16	4.26 ± 0.16	4
TYR	5.62 ± 0.04	5.08 ± 0.13	5.71 ± 0.14	5.57 ± 0.25	5
PHE	0	0	0	0	0
HIS	0.76 ± 0.03	0.58 ± 0.04	1.03 ± 0.17	0.95 ± 0.11	1
TRP	0.72 ± 0.18	0.81 ± 0.17	0.92 ± 0.46	0.71 ± 0.20	2
LYS	0	0	0	0	0
ARG	4.86 ± 0.15	4.95 ± 1.11	4.45 ± 0.06	4.55 ± 0.17	4
CYS[c]	5.48 ± 0.08	5.80 ± 0.02	5.17 ± 0.28	4.91 ± 0.31	6
PRO	2.26 ± 0.01	2.32 ± 0.32	2.50 ± 0.14	2.44 ± 0.24	2

[a]Data are expressed as residues per molecule based on a molecular weight of 6045. Values are means ± standard deviations of 2 (male EGF) or 4 (female EGF) determinations and are not corrected for hydrolysis losses.
[b](Savage et al., 1972) [c]Determined as cysteic acid.

by about 30% (from 728 to 533 μg). This reduction in EGF yield could be prevented by incubating the supernatant for 60 min at pH 1.0 prior to application to the chromatography system. No change of the retention time of the immunoreactive material was observed when the acidification step was omitted. When saliva was treated with acid in an analogous manner prior to RPLC, different chromatograms were also observed (not shown). However, no difference in yield of immunoreactive EGF I and II occurred.

Structural analysis of EGF I and II- The EGFs isolated from saliva of male and female mice with the techniques described above were analyzed for amino acid composition (Table I) and NH_2-terminal primary structure (Table II). Amounts of 10 to 20 pmol of EGF I and II were hydrolyzed and used for amino acid analysis, amounts of 500 pmol (male) and 860 pmol (female) EGF I and 700 pmol (male) and 400 pmol (female) EGF II were applied to the sequencing system. The amino acid composition obtained revealed differences in aspartic acid contents between EGF I and EGF II. Microsequencing revealed that EGF I from male and female saliva started with an asparagine residue (Table II; data for

Table II. **Microsequence analysis of male saliva EGF I and EGF II**

	EGF I[a]		EGF II[b]	
Cycle	Residue	Pmoles	Residue	Pmoles
1	Asn[c]	105	Ser[c]	74
2	Ser[c]	25	Tyr	236
3	Tyr	83	Pro	150
4	Pro	67	Gly	215
5	Gly	44	(Cys)	-
6	(Cys)	-	Pro[c]	151
7	Pro[c]	48	Ser[c]	58
8	Ser[c]	20	Ser[c]	65
9	Ser[c]	32	Tyr	159
10	Tyr	27	Asp	105
11	Asp	5	Gly	138
12	Gly	16	Tyr	176
13	Tyr	15	(Cys)	-
14	(Cys)	-	Leu	246
15	Leu	26	Asn	59
16	Asn	14	Gly	79
17	Gly	15	Gly	92
18	Gly	20	Val	105
19	Val	11	(Cys)	-
20	(Cys)	-	Met	41
21	Met	6	His	10
22	His	1	Ile	75
23			Gln[c]	37
24			Ser[c]	15
25			Leu	106
26			(Asp)	(10)
27			Ser[c]	13
28			Tyr	55
29			Thr[c]	15
30			(Cys)	-
31			Asn	6
32			(Cys)	-
33			Val	33

[a]Approximately 500 pmoles of sample was sequenced with an initial yield of 21% (based on cycle 1). Cysteine residues do not give identifiable derivatives and are shown in brackets.

[b]Approximately 700 pmoles of sample was sequenced with an initial yield of 34% (based on cycle 2). The aspartic acid residue at cycle 26 was only tentatively identified (shown in parenthesis).

[c]Serine and threonine residues give two to three derivatives, only one of which is reported here.

female peptides not shown). In contrast, the N-terminal sequence of male and female EGF II began with serine. Cysteine residues were not positively identified (for instance by making the S-alkylated derivatives) in this analysis, but no other residues were identified at cycles 5, 13, 19, 30 and 32. Based on this analysis the partial sequence determined of male EGF II corresponds exactly to residues 2 - 34 of the sequence of EGF I.

DISCUSSION

Our studies demonstrate that the isolation of immunoreactive EGF from submaxillary glands by an approach which differs considerably from the classical method leads to the identification of two EGF species. The present isolation procedure allows the purification of the murine EGFs to homogeneity - as judged by amino acid analysis (absence of alanine, phenylalanine and lysine residues) and by NH_2-terminal sequence analysis - within hours. Utilizing a similar approach these molecules can also be easily isolated from saliva. Semipreparative RPLC essentially allows the single step purification of EGF I and Des-Asn-EGF I (EGF II) with yields of 4.2 and 1.4 nmol resp. from 0.2 ml male saliva. Interestingly, lack of treatment with strong acid (pH 1.0) of the tissue extract prior to application (either by redissolving the lyophilizate in acid or preincubation of the supernatant in hydrochloric acid) significantly decreases the yields of immunoreactive EGF. With saliva preparations, however, no difference in EGF yield is observed whether or not the fluid is pretreated with strong acid. In conjunction with the observation that no change in retention time of the immunoreactive peaks occured this suggests the presence of a proteolytic activity which (1) is active at pH 3.0, (2) is inactivated by pH reduction to 1.0 and (3) converts EGF into a product which is not detectable in the radioimmunoassay any longer. Apparently, this activity is only present in submaxillary glands extracts but not in saliva, which makes it likely to be of lysosomal origin. The observation probably also explains the high yields of the classical EGF isolation procedure which gave - despite the fact that adsorption phenomena play a major role - much better yields than the methods previously used by these authors which did not employ low pH values. Amino acid analyses of the two EGFs revealed a quantitative difference in aspartic acid residues between the two species from male as well as female sources. Moreover, the observation that EGF I and II showed UV-absorption equivalent to their amount as measured by radioimmunoassay but different fluorescence yields when monitored with the postcolumn fluorescence derivatization system (Fig. 3) made the NH_2-terminal portion a candidate region for the difference because of the lack of ϵ-amino groups for the reaction with fluorescamine in EGF. Sequence analysis of both molecules at the subnanomol level confirmed the lack of the NH_2-terminal asparagine residue in EGF II as the difference between the two

molecules. Apparently, the fluorescent yields of the derivatization reaction with fluorescamine differ for EGF I starting with an asparagine residue and EGF II beginning with a serine residue.

We do not know whether EGF II is produced directly from the large EGF-precursor (27,28) or by further processing of EGF I. In any case, an enzyme which cleaves at the C-terminal of asparagine could be responsible for the generation of EGF II.

At present, the physiological significance of the existence of two EGFs in submaxillary glands and saliva is unclear. According to earlier studies (12) the two molecules are equipotent in in vivo (eyelid opening) and in vitro (^{3}H-thymidine incorporation in cell culture) assays. Thus, the structural difference may have other implications such as upon the the formation or dissociation of the complex between EGF and its binding protein: at neutral pH the EGF molecule in submaxillary glands and saliva is noncovalently associated with a larger protein with arginine esterase activity (29). This high molecular complex is dissociated by altering the pH into the acidic or alkaline range. Although it has been reported that the carboxy-terminal portion of EGF is essential for the formation of the complex (30), nothing is known about a possible importance of the amino-terminal portion. Recently, the EGF binding protein has been separated into two sequence variants, types A and B (31). From the existence of two low molecular weight EGFs and two EGF binding protein variants one has to postulate the presence of several species of high molecular weight EGF complexes which may differ in their dissociation constants for instance.

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