

Epidermal Growth Factor: High and Low Molecular Weight Forms

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Abstract. In crude homogenates of the submaxillary gland of the male mouse, epidermal growth factor (EGF) activity was found almost entirely in a high molecular weight complex (about 74,000 daltons). This high molecular weight form may be reversibly dissociated into EGF and an EGF-binding protein. EGF was found to have a molecular weight of approximately 6400. The EGF-binding protein has a molecular weight of 29,000, and shows arginyl-esterase activity.

In 1962, Cohen¹ reported the isolation of a polypeptide from the submaxillary gland of male mice that accelerated eyelid opening and incisor eruption in the newborn animal. This polypeptide, epidermal growth factor (EGF), was subsequently found to stimulate proliferation and keratinization of mouse epidermis *in vivo*² and the growth of chick embryo epidermis *in vitro*.³ The metabolic events that follow the addition of EGF to cultures of chick embryo epidermis include: the stimulation of protein and RNA synthesis,⁴ the formation of ribosomes which are more active in cell-free protein synthesis,⁵ the conversion of pre-existing ribosomal monomers into polysomes,⁶ the induction of ornithine decarboxylase,⁷ and the accumulation of polyamines.⁷ EGF also has been reported to stimulate the growth of mammary epithelial cells in organ culture,⁸ and to stimulate the accumulation of triglycerides in the liver of the intact animal.⁹

In this report evidence will be presented which suggests the molecular weight of EGF to be approximately 6400. However, in crude homogenates of the submaxillary gland of the male mouse, EGF exists almost entirely in a high molecular weight complex (about 74,000 daltons). This high molecular weight form may be reversibly dissociated into EGF and a protein of molecular weight 29,000. This EGF-binding protein is an esterase with an apparent high degree of specificity for arginine esters.

Materials and Methods. Submaxillary glands were excised from adult male albino mice, and stored frozen at -20°C until used.

Gel filtration was performed at 4°C by the reverse-flow technique using Sephadex G-100 (Pharmacia). The columns were calibrated as described by Whitaker.¹⁰ Ion-exchange chromatography was performed with DE 52 cellulose and CM 52 cellulose (Whatman). All column eluates were monitored by an ISCO UV flow monitor at 280 nm. Protein content was estimated by the procedure of Lowry *et al.*¹¹ using bovine serum albumin (Armour) as a standard. Antiserum to EGF was prepared as previously described.¹

Polyacrylamide gel disc electrophoresis was performed on Canalco equipment according to the manufacturer's directions¹² and the method of Davis¹³ using a pH 9.3 resolving gel system. Isoelectric focusing was performed on LKB equipment according to the manufacturer's directions¹⁴ and as described by Haglund¹⁵ at 5°C.

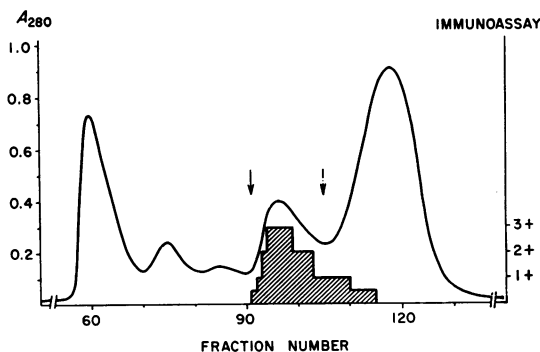
Ultracentrifuge studies were performed with a Spinco model E analytical ultracentrifuge equipped with Rayleigh interference optics using double sector cells and quartz windows. A Nikon two-dimensional microcomparator was used to analyze the photographic plates. All analyses were run at 15°C using samples that had been exhaustively dialyzed against pH 5.6 sodium acetate 0.10 M. Sedimentation equilibrium by the meniscus-depletion method was performed according to Yphantis.¹⁶ Low-speed sedimentation equilibrium was performed as described by Mitchell and Hash.¹⁷ In all cases, the ultracentrifuge was allowed to run for 24 hr at the equilibrium speed. It was considered that equilibrium conditions had been reached when the fringe concentration gradient showed no change with sequential photographs.

Amino acid analysis was done according to the method of Spackman, Stein, and Moore¹⁸ on a Spinco model 120 C analyzer, equipped with an Infotronics integrator. Tryptophan was determined according to the spectrophotometric method of Goodwin and Morton.¹⁹

Results. Preparation of high molecular weight EGF (HMW-EGF): The initial steps in the purification of high molecular weight EGF were essentially identical to those previously described for low molecular weight EGF.¹ Approximately 20 g wet weight of submaxillary glands from 150 mice were homogenized and carried through the streptomycin and ammonium sulfate precipitation steps. However, the water-dialyzed preparation at this point was not heated, but lyophilized, and carried through the following procedure.

(a) *Gel filtration on Sephadex G-100:* The dry powder was dissolved in 40 ml of cold 0.01 M sodium acetate at pH 5.9 containing 0.1 M sodium chloride. The concentrated extract was centrifuged at 17,500 rpm for 3 hr and the residue discarded. Half of the supernatant, (0.9–1.0 g protein) was applied to a Sephadex G-100 column equilibrated with the same buffer. A typical fractionation is reported in Figure 1. The EGF-antibody precipitating peak emerged just slightly ahead of and in the leading portion of the pigmented hemoglobin peak. The fractions between the arrows in Figure 1, when combined, contained approximately 150 mg of protein.

FIG. 1.—Gel filtration of male mouse submaxillary gland extract. 20 ml of extract containing 1 g of protein was applied to a Sephadex G-100 column (5.0 × 90 cm) equilibrated with 0.01 M sodium acetate pH 5.9 containing 0.1 M NaCl. The flow rate was 2.0 ml/cm² per hr and 10-ml fractions were collected. The hatched area indicates the immunoprecipitation reaction of the eluate with antibody to low molecular weight EGF. The immunoassay was carried out by layering 25 μ l from each tube of eluate over 25 μ l of antiserum and qualitatively observing the precipitate that formed at the interface after 30 min. The fractions between the arrows were combined for further purification.



(b) *Ion-exchange chromatography of the G-100 material:* The combined fraction was concentrated in a Diaflo ultrafiltration cell to approximately 10 ml. The pH of this material was lowered to pH 5.1 by pressure dialysis with 0.01 M acetate buffer, pH 5.1. The sample was then reconcentrated to 10 ml and centrifuged at 2500 rpm for 10 min. The supernatant was applied to a small column of DE 52 cellulose equilibrated with 0.01 M sodium acetate, pH 5.1, and eluted with the same buffer. The EGF-active material under these conditions does not adsorb to the resin. The pH of the eluate (containing approximately 100 mg protein) was adjusted to pH 5.4 with 0.05 M sodium acetate. The sample was concentrated to 10 ml and applied to a small column of CM 52 cellulose equilibrated with pH 5.4 0.05 M sodium acetate buffer, and eluted with the same buffer. Again, the EGF-active material passed immediately through the resin without adsorption. The eluate at this point contained approximately 50 mg of protein. After concentration, it showed only one band on cellulose acetate electrophoresis at pH 7.0 using 0.02 M citrate buffer. As will be shown later, this protein had a molecular weight of approximately 74,000, and was biologically active.¹ It was termed high molecular weight EGF (HMW-EGF) and represented approximately 2-3% of the dry weight of the submaxillary gland. The above conditions for the isolation of HMW-EGF were dictated by the observation that it is readily dissociated upon adsorption to a variety of ion-exchange support media.

Subunit character of high molecular weight EGF: HMW-EGF can be dissociated into the low molecular weight EGF and an EGF-binding protein by adsorption to pH 7.5 DE 52 cellulose followed by elution with a sodium chloride gradient. HMW-EGF obtained from the CM 52 cellulose column was brought to pH 7.5 with 0.02 M Tris·HCl and applied to a column of DE 52 cellulose equilibrated with the same buffer. The HMW-EGF, under these conditions, is adsorbed to the column and can be subsequently eluted with a salt gradient prepared by allowing pH 7.5 Tris·HCl, 0.02 M, containing 0.1 M NaCl to flow into a 110 ml constant-volume mixing chamber containing the salt-free buffer. A typical elution pattern is given in Fig. 2. The last peak to be eluted was found to be identical to the low molecular weight EGF as originally isolated by Cohen.¹ Criteria of identity included amino acid analysis, isoelectric point of

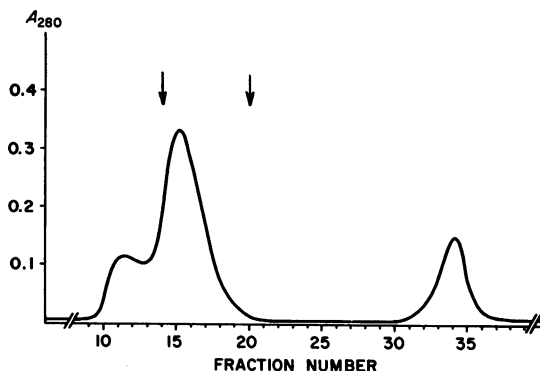


FIG. 2. Ion-exchange chromatography of high molecular weight EGF. 10 ml of the CM 52 cellulose eluate containing 50 mg of HMW-EGF were applied to a 1.5×25 cm column of DE 52 cellulose equilibrated with pH 7.5 Tris·HCl, 0.02 M. The protein was eluted with a gradient of 0-0.1 M NaCl in this same buffer (see text for details). The material between the arrows was found to be an EGF-binding protein.

pH 4.6 by the method of isoelectric focusing,¹⁴ and antigenic identity against antibody prepared from EGF as originally prepared by Cohen.¹ It was the only material eluted that formed a precipitate to the EGF antibody.

The material between the arrows in Fig. 2 was found to bind the low molecular weight EGF as described in the following section. The purity of the EGF-binding protein and EGF was checked on polyacrylamide gel disc electrophoresis according to the method of Davis,¹³ as shown in Fig. 3. The nature of the first peak, shown in Fig. 2, is under study.

High molecular weight EGF can also be dissociated into low molecular weight EGF and EGF-binding protein by isoelectric focusing in an acid pH range as shown in Fig. 4. Only two major protein peaks are observed above the back-

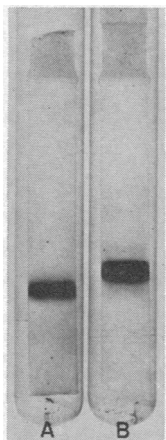


FIG. 3. Disc gel electrophoresis of EGF-binding protein and low molecular weight EGF. *Tube A* contains 50 μ g of EGF and *Tube B* contains 30 μ g of EGF-binding protein. The pH of the resolving gel was pH 9.3. The cathode was at the top of the column.

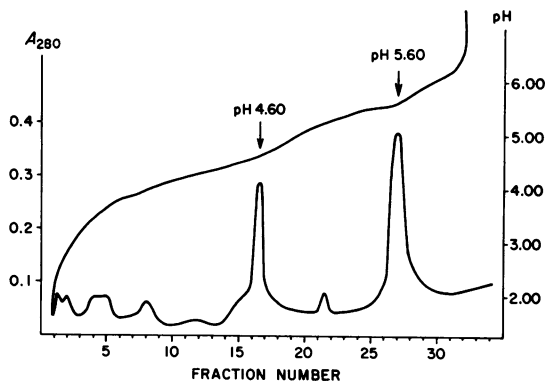


FIG. 4. Isoelectric focusing of high molecular weight EGF. 8 mg of HMW-EGF eluted from the CM 52 cellulose was added to a pH 4–pH 6 range ampholyte solution. Fractions of 2 ml were collected and the pH of each fraction was measured with a Radiometer pH meter.

ground absorbance. The peak at pH 4.6 corresponds to low molecular weight EGF and the peak at pH 5.6 corresponds to the EGF-binding protein. No evidence of any other protein was observed at either end of the isoelectric focusing column.

Recombination of EGF-binding protein and EGF: Equal weights of EGF-binding protein and EGF obtained from the pH 7.5 DE 52 cellulose column were mixed together in pH 5.9 sodium acetate, 0.01 M, containing 0.1 M NaCl. After 4 hr, the mixture was concentrated by ultrafiltration and then applied to a column of Sephadex G-100. The column had been previously calibrated with various known marker proteins,¹⁰ EGF-binding protein, and low molecular weight EGF. The results of the recombining experiment are shown in Figure 5. The

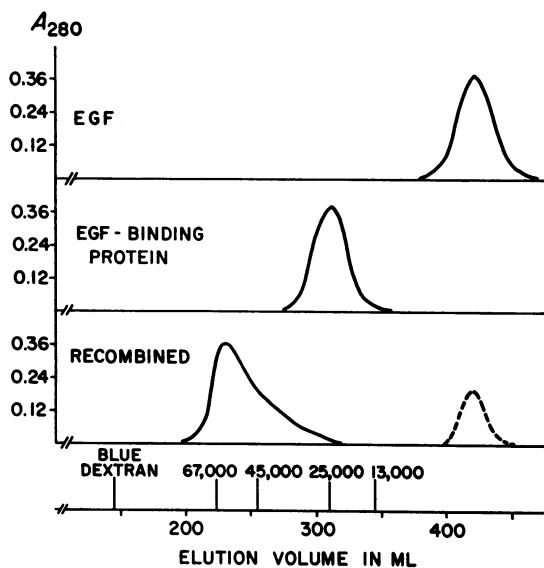


FIG. 5. Recombination of EGF-binding protein and low molecular weight EGF. A 4-ml sample containing protein was applied to a 2.5×90 cm Sephadex G-100 column equilibrated with 0.01 M sodium acetate pH 5.9 containing 0.1 M NaCl. Flow rate was 4.3 ml/cm² per hr. The top curve shows the elution of pure low molecular weight EGF; the middle curve shows the elution of pure EGF-binding protein; and the bottom curve shows the elution of the recombined EGF (the dotted line indicates excess unreacted low molecular weight EGF). At the bottom of the figure is shown the elution volumes and molecular weights of various marker proteins: bovine serum albumin, ovalbumin, chymotrypsinogen, and horse heart cytochrome c.

EGF-binding protein alone did not form a precipitate with the EGF antibody, whereas the recombined EGF did form a precipitate. In addition, the elution volume of the recombined EGF was similar to that of the isolated original high molecular weight EGF, and distinctly different from the elution volumes of its two components.

Ultracentrifugation of HMW-EGF and EGF-binding protein: The sedimentation equilibria of HMW-EGF and recombined HMW-EGF were examined with the model E analytical ultracentrifuge using the meniscus-depletion method of Yphantis.¹⁶ The ultracentrifuge was run at 23,150 or 25,980 rpm under the conditions described in *Materials and Methods*. The molecular weights were found to be 74,800 and 72,200, respectively, indicating a close similarity in molecular size.

The sedimentation equilibrium behavior of the EGF-binding protein was examined using the low-speed method¹⁷ at 12,590 rpm. The molecular weight was found to be 29,000. Under the conditions described, each protein examined in the ultracentrifuge showed no significant heterogeneity.

Molecular weight of low molecular weight EGF: A preparation of EGF as originally isolated by Cohen¹ was analyzed by low-speed sedimentation equilibrium, using Rayleigh interference optics, at 31,310 rpm and was found to have a molecular weight of 6400. Linear plots of log concentration versus r^2 revealed no significant heterogeneity.

The results of amino acid analysis, as shown in Table 1, indicate a minimum molecular weight of approximately 6400. EGF is characterized by the absence of three specific amino acids: lysine, phenylalanine, and alanine. Low molecular weight EGF as isolated from HMW-EGF showed an identical amino acid analysis, further documenting its identity with Cohen's original EGF.

Immunological studies: Rabbit antiserum was prepared against Cohen's

TABLE 1. Amino acid composition of low molecular weight EGF.

Amino acid	Residues per mole	Amino acid	Residues per mole
Lysine	0	Alanine	0
Histidine	1	Half-cystine	6
Arginine	4	Valine	2
Aspartic acid	8-9	Methionine	1
Threonine	2	Isoleucine	2
Serine	6-7	Leucine	4
Glutamic acid	3-4	Tyrosine	5
Proline	2	Phenylalanine	0
Glycine	6-7	Tryptophan	2
Total residues		54-58	
Minimum molecular weight		6166-6554	

Samples of 100 μ g were hydrolyzed in 6 N HCl under vacuum for 24 hr. The average value from two hydrolyses is presented. The calculated number of residues was based on one histidine and four arginine residues per mole of polypeptide. The results were uncorrected for hydrolytic losses. The recovered amino acids amounted to 85% by weight of the starting material. Tryptophan was measured spectrophotometrically.¹⁹

original low molecular weight EGF as previously described.¹ By means of immunodiffusion in agarose gels, the reaction of the antiserum to each of the following proteins was examined: HMW-EGF, recombined HMW-EGF, EGF-binding protein, EGF isolated according to Cohen,¹ EGF isolated from HMW-EGF, and crude submaxillary gland homogenate. The result is shown in Fig. 6. The EGF-binding protein did not form a precipitin band, whereas the precipitin bands of the remaining samples form a line of identity indicating a close antigenic similarity between a portion of the high molecular weight EGF and the low molecular weight form.

Discussion. The high molecular weight epidermal growth factor (HMW-EGF) was isolated utilizing its ability to form a precipitate with antibody prepared against the low molecular weight factor (EGF). HMW-EGF has been purified approximately 40 to 50 times from the initial homogenates of the submaxillary glands of adult male mice. Because of the problems inherent in the biological assay, as discussed by Cohen,¹ it is difficult to obtain exact data on the specific activity of the HMW-EGF at each stage of the purification. When the bioassay is carried out according to Cohen,¹ HMW-EGF has approximately one-sixth the biological activity of an equal weight of the low molecular weight EGF when injected subcutaneously into the newborn animal. Furthermore, HMW-EGF shows a dose response in this assay similar to EGF.¹ These observations agree with the physical data which suggest that low molecular weight EGF is one-sixth, by weight, of the HMW-EGF complex. It is tentatively felt

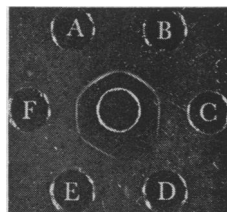


FIG. 6. Immunodiffusion pattern of EGF proteins. The center well contains antiserum to the low molecular weight EGF.¹ The outside wells contain: (A) EGF prepared according to Cohen,¹ (B) EGF isolated from the high molecular weight EGF complex; (C) crude homogenate; (D) EGF-binding protein; (E) recombined high molecular weight EGF; and (F) high molecular weight EGF.

that 2 EGF molecules and 2 molecules of EGF-binding protein combine to form the larger complex.

In this communication we have reported the molecular weight of EGF, as isolated by Cohen,¹ to be approximately 6400. This number differs from the estimate of 15,000 as originally reported in 1962.¹ The 1962 estimate, however, was calculated from the amino acid composition on the basis of one alanine residue and 10 leucine residues per mole. Recent amino acid analyses of EGF, prepared under improved conditions which reduce contamination, do not reveal the presence of a significant quantity of alanine. Furthermore, the sedimentation equilibrium data, as reported here, support the lower molecular weight estimate.

After the HMW-EGF had been isolated, the observation was made that the EGF-binding protein subunit (molecular weight 29,000) catalyzed the hydrolysis of benzoyl-arginine ethyl ester.²⁰ The rate of hydrolysis of this ester at 25°C was approximately 390 $\mu\text{mol}/\text{min}$ per mg of enzyme at pH 8. In a preliminary investigation of its enzymatic activity, the EGF-binding subunit catalyzed the hydrolysis of benzoyl-lysine methyl ester to a lesser degree, with no detectable hydrolysis of benzoyl-arginine amide. HMW-EGF and recombinated HMW-EGF also catalyzed the hydrolysis of benzoyl-arginine ethyl ester. The additional observation that EGF possesses a C-terminal arginine²⁰ suggests that EGF may be generated from a precursor protein via the possible proteolytic action of the EGF-binding esterase.

In broader terms, the observations that certain other polypeptide hormones such as bradykinin²¹ and insulin²² arise from precursors via the proteolytic action of arginine esterases, and the report²³ that the mouse submaxillary nerve growth factor, originally isolated by Cohen,²⁴ can be found associated with an arginine esterase, suggest that the formation of active polypeptide hormones from inactive precursors, by the proteolytic action of a family of arginine esterases, may be a general phenomenon.

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Abbreviations used: EGF, epidermal growth factor; HMW-EGF, high molecular weight EGF.

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A preliminary report of this work has been presented.²⁰

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