dicted HO concentration relative to that for the natural troposphere (*21*). Therefore, the observation of a peak HO concentration greater than 10^7 molecule cm^{-3} would suggest the presence of an efficient, direct photochemical source of HO. A plausible mechanism involves HONO (*22*), which generates HO upon dissociation by the solar radiation. The importance of this reaction in smog formation (*23*) has been recognized for some time. Estimates based on the absorption cross section of HONO indicate that concentrations in the parts-per-billion range would lead to the generation of excess HO concentrations, sufficiently high to explain the enhanced peak values observed here, and may also explain the occurrence of a second HO peak in the late afternoon. Work is in progress to ascertain the relevance of this mechanism.

To summarize, diurnal variations in the HO concentration of ambient air were measured for the first time by the technique of laser-induced fluorescence. The HO concentration builds up in the morning, peaks in the afternoon, and decreases to below the detection limit at night. The peak HO concentration also varies from day to day; it reaches a level in excess of 10^7 molecule cm^{-3} on sunny summer days but is below the detection limit on rainy or cloudy days. Our results indicate that, in addition to processes operative in the natural troposphere that produce HO, direct production of HO through photodissociation of atmospheric contaminants such as HONO may also play an important role in determining the HO concentration observed in our measurements.

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14. The photolysis reaction is
$$HONO + h\nu\ (\lambda = 2900 \text{ to } 4000\ \text{Å}) \rightarrow HO + NO$$
where $h\nu$ is a photon. H. S. Johnston and R. Graham, *Can. J. Chem.* **52**, 1415 (1974); R. A. Cox, *J. Photochem.* **3**, 175 (1974).
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20. A major atmospheric source of HO involves the photolysis of O_3
$$O_3 + h\nu\ (\lambda < 3130\ \text{Å}) \rightarrow O(^1D) + O_2$$
followed by
$$O(^1D) + H_2O \rightarrow 2HO$$
21. The effect of a higher concentration of CO is to shift the ratio of $[HO_2]/[HO]$ to a higher value by the two reactions:
$$CO + HO \rightarrow CO_2 + H$$
and
$$H + O_2 + M \rightarrow HO_2 + M$$
followed by the decomposition of HO_2
$$2HO_2 \rightarrow H_2O_2 + O_2$$
The net effect is a reduced concentration of HO. At higher NO_2 concentrations, the reaction
$$HO + NO_2 + M \rightarrow HNO_3 + M$$
is a major removal mechanism of HO.
22. The formation of HONO in the atmosphere may occur according to the reactions
$$NO + NO_2 + H_2O \rightleftharpoons 2HONO$$
and
$$HO + NO + M \rightarrow HONO + M$$
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6 May 1975

Epidermal Growth Factor: Identification of a New Hormone in Human Urine

Abstract. *Epidermal growth factor is a polypeptide hormone that was previously identified only in mice. It causes proliferation and keratinization of epidermal tissues. An immune affinity column extraction technique was used to purify partially a substance from human urine which was similar to the mouse hormone in both its biological activity and immunoreactivity.*

Epidermal growth factor (EGF) is a polypeptide hormone originally isolated from the submaxillary glands of adult male mice. Its discovery was a result of the observation that extracts of these glands caused precocious eye opening and tooth-bud eruption when injected into immature mice (*1*). These morphological effects are now known to be due to stimulation of epidermal growth and keratinization (*2*).

Since its discovery in 1962, considerable knowledge has accumulated about mouse EGF, much of which has been summarized (*3*). Mouse EGF is both heat and acid stable and has a molecular weight of 6045; its primary chemical structure is known (*4*). The metabolic events that occur when epidermal cells are exposed to EGF include stimulation of ornithine decarboxylase activity, accumulation of polyamines, and increased synthesis of RNA and protein (*5*). Initially, the biological assay for EGF was based on early eye opening in the immature mouse. Further elucidation of EGF physiology was made possible by the subsequent development of a sensitive and specific radioimmunoassay (*6*). In the mouse, submaxillary gland EGF concentration is dependent on androgen stimulation, but plasma EGF levels are not androgen dependent. Mouse urine, milk, and saliva contain much higher concentrations than does plasma. Alpha-adrenergic stimulation causes release of submaxillary gland EGF, resulting in marked increases in plasma EGF concentrations, and alpha-adrenergic blockade abolishes this response. Neither beta-adrenergic stimulation nor blockade has any effect. When the submaxillary glands are removed, the response of plasma EGF to alpha-adrenergic stimulation is abolished, yet basal plasma EGF concentrations are unchanged (*7*). This suggests that there may

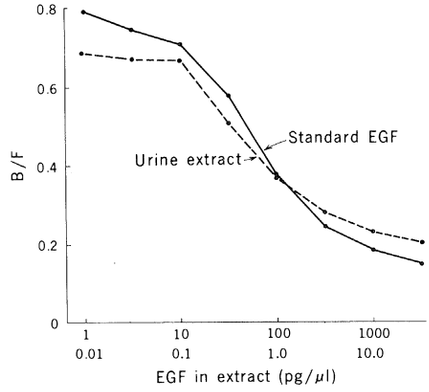

Fig. 1. Competitive binding curves of mouse EGF and affinity column extract of human urine. B/F is the ratio of antibody bound to free ^{125}I-labeled mouse EGF.

be other sites of EGF synthesis in the mouse and, indeed, EGF can be found in significant concentrations in a variety of other tissues after the submaxillary glands have been excised (*6*).

EGF has not previously been isolated from species other than the mouse. Two lines of evidence, however, suggested that this hormone might exist in man. First, mouse EGF has been found to be biologically active in human tissue, stimulating (i) epithelial growth in cultured human fetal cornea and epidermis (*8*) and (ii) DNA and RNA synthesis in cultured human fibroblasts (*9*). Receptors capable of binding labeled mouse EGF have been found on human fibroblasts (*9*). Second, we have found low levels of EGF immunoreactivity in human urine and milk, although none is usually detectable in human plasma (*10*). The ready availability of human urine and the knowledge that mouse urine contains much more EGF than mouse plasma prompted us to attempt to extract EGF from human urine. We report here the partial purification of immunologically reactive and biologically active EGF from human urine.

Affinity chromatography columns were prepared by covalently linking rabbit antiserum to mouse EGF (*6*) to cyanogen bromide–activated agarose beads (CNBr-activated Sepharose 4B, Pharmacia) by the method of Gospodarowicz (*11*). Preliminary experiments demonstrated that a column (0.8 by 1.0 cm) could be used to extract mouse EGF that was added to 700 ml of human urine. The urine was adjusted to *p*H 7.3 with NaOH, and ^{125}I-labeled mouse EGF (1 to 2 pg/ml) was added. The urine was passed over the column at a rate of 40 ml/hour at room temperature. The column was then washed extensively with phosphate-buffered saline, *p*H 7.3. Adsorbed EGF was eluted with 10 ml of 0.55*M* formic acid with recovery of 70 to 85 percent of the labeled EGF. There was no certainty, of course, that human EGF would be extracted with similar efficiency.

Pooled human urine (up to 10 liters) was then passed over a larger column (2.5 by 2.0 cm), the increased cross-sectional area of which permitted faster flow rates. After elution with formic acid, the extract was lyophilized, dissolved in distilled water, and dialyzed against Hanks' solution to remove residual formic acid. The dialyzate was then tested for immunoreactivity and biologic activity.

Radioimmunoassays were performed as described (*6*) with two modifications. The standard diluent for the incubation mixtures was 50 m*M* phosphate, 77 m*M* NaCl buffer, *p*H 7.4, containing merthiolate (0.1 mg/ml) to inhibit bacterial growth, egg white lysozyme (1 mg/ml) as a carrier for antigen, and human plasma (1 percent) as a carrier for the immunoglobulin G (IgG) fraction of antiserum to EGF. Polyethylene glycol (Carbowax 6000, Union Carbide) was used to separate bound from free hormone (*12*). This radioimmunoassay used rabbit antibody to mouse EGF and mouse EGF as ^{125}I-labeled tracer and unlabeled standard. The competitive binding curves for standard mouse EGF and for the human urine extract in the EGF radioimmunoassay are shown in Fig. 1. The urine extract yielded a curve similar to that of mouse EGF, suggesting that the substances are themselves immunochemically similar.

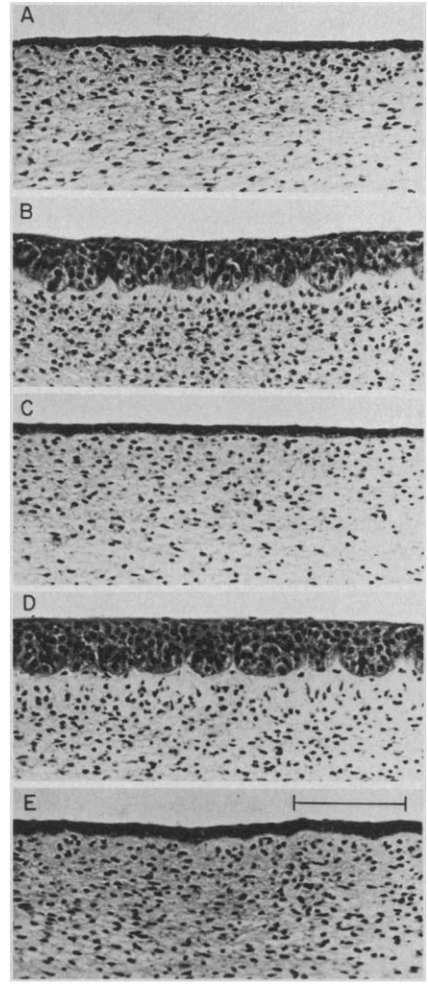

Fig. 2. Histologic sections of chick embryo cornea after 4 days of incubation in organ culture under the following conditions: (A) control with culture medium only; (B) with mouse EGF, 200 ng/ml; (C) with mouse EGF and IgG fraction of mouse antiserum to mouse EGF; (D) with human urine extract; (E) with human urine extract and the IgG fraction of mouse antiserum to EGF. The darkly stained layer of epithelial cells can be seen on the upper surface of the more lightly stained corneal stroma in each section. The epithelial layer is one to two cells thick in the control tissue (A) and six to eight cells thick in tissues treated with either mouse (B) or human (D) EGF; this effect is neutralized by addition of IgG fraction of mouse antiserum to EGF (C and E). Scale bar, 100 μm (same for each photomicrograph).

Biologic activity was assessed in an assay based on histologic evidence of proliferation of chick embryo corneal epithelium in organ culture (*8*). Forty nanograms of mouse EGF produced marked epithelial proliferation in this assay, but 150- to 500-fold greater amounts of adrenocorticotrophic hormone, follicle-stimulating hormone, thyroid-stimulating hormone, human chorionic gonadotrophin, growth hormone, and prolactin had no effect (*13*). In concentrations of micrograms per milliliter, insulin caused minimal proliferation, an effect which was not inhibited by the IgG fraction of mouse antiserum to EGF prepared by DEAE-cellulose chromatography (*14*). This presumably represented the growth-promoting effect of insulin observable in many tissues (*15*). Figure 2 compares the stimulation of growth produced in the EGF bioassay by mouse EGF and by the human urine extract and demonstrates the inhibition of growth which resulted when the IgG fraction of mouse antiserum to EGF was added to the respective culture mediums. These observations indicate that the substance extracted from human urine is biologically similar to mouse EGF and that antibodies to mouse EGF are capable of neutralizing its biologic effect. Unfortunately, the bioassay does not lend itself readily to large numbers of determinations or to precise quantitation of the hormone.

Experiments were performed to assess the specificity of the adsorption and elution procedures. Buffer passed through the column packed with agarose bound to rabbit antiserum to mouse EGF or human urine passed through a column packed with agarose bound to serum from an unimmunized rabbit had neither biological activity nor immunological reactivity. When urine which failed to yield active material by nonimmune column extraction was subsequently passed over the EGF immune column, bioactive and immunoreactive material was recovered.

Using this affinity chromatography technique, we have recovered human EGF from the urines of pregnant women, nonpregnant women, and men. The amount of EGF detected in an extract by radioimmunoassay appeared to be 1000 times less than was detected by bioassay. While it is possible that human EGF is biologically more potent than mouse EGF, it seems to us more likely that human EGF is less reactive with the antibodies to mouse EGF than is mouse EGF itself. Thus, the quantity of human EGF required to produce a given degree of displacement of labeled mouse EGF from the antibodies may be three orders of magnitude greater than the quantity of mouse EGF necessary to produce the same amount of displacement. The present radioimmunoassay may detect

only extremely high levels of human EGF and may greatly underestimate their true values. We have not found immunoreactivity in the plasma of normal adult humans. Ances (*16*), using the same antiserum and EGF standard as we did in our assay but only one dilution of serum, has reported immunoreactive EGF levels of 0.5 to 6.0 ng/ml in serum from pregnant women. While these levels are well within the range of detectability of our assay, we have been unable to confirm these findings. We have, however, detected low levels of immunoreactivity (100 to 300 pg equivalents of mouse EGF per milliliter) in the plasma of newborn infants, suggesting that the levels in newborns may actually be quite high. In most of the adult urine specimens we have assayed, we were able to detect the equivalent of 50 to 400 pg/ml of immunoreactive mouse EGF. Human saliva and extracts of human submaxillary glands, prepared as described (*1*), did not contain detectable concentrations of immunoreactive EGF, suggesting that the salivary glands are probably not the source of the hormone in humans.

Thus, we have detected in human urine a material that is biologically and immunologically similar to EGF. Once a sensitive and specific radioimmunoassay for human EGF is developed, it should be possible to measure accurately minute amounts of EGF in human tissues and fluids to elucidate the role of this new human hormone in health and disease.

Addendum. Since this manuscript was submitted for publication, highly purified human EGF has been obtained from urine by means of an improved extraction technique, monitored with the aid of a new radioreceptor assay for EGF. The amino acid composition and estimated molecular weight of human EGF indicate that it is similar to, but biochemically distinct from, mouse EGF (*17*). On the basis of comparisons of radioimmunoassay and radioreceptor assay results, we have also confirmed that human EGF is about three orders of magnitude less reactive with antibodies raised to mouse EGF than is mouse EGF itself (*18*). Thus, the mouse EGF radioimmunoassay does greatly underestimate levels of human EGF.

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16 December 1974; revised 21 March 1975

Chemotaxis away from Uncouplers of Oxidative Phosphorylation in *Bacillus subtilis*

Abstract. *In a capillary assay, uncouplers of oxidative phosphorylation and inhibitors of electron transport are repellents for* Bacillus subtilis. *They also cause transient tumbling in naturally smooth swimming strains. Tumbling strains can be made to swim smoothly by addition of attractant and then immediately returned to tumbling by subsequent addition of repellent. Arsenate does not cause transient tumbling, suggesting that decrease in concentration of adenosine triphosphate does not cause tumbling and that adenosine triphosphate concentration does not govern tumbling frequency. Instead, the evidence suggests that diminution of the energized state of the membrane, or membrane potential, causes tumbling although the level of the energized state itself does not govern tumbling frequency.*

Chemotaxis is the process by which motile bacteria swim to higher concentrations of attractant or lower concentrations of repellent. *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilis* normally swim smoothly but randomly change direction by tumbling (*1*). When headed to higher concentrations of attractants (for example, some amino acids and sugars) the bacteria tumble less often than in isotropic medium; when headed to lower concentrations, they show at most a slight tendency to tumble more often (*1, 2*). Behavioral changes can be observed directly by adding attractant or repellent to a bacterial suspension and observing transient decreases or increases in tumbling frequency (*1, 3*). Chemotaxis, then, is caused by modulation of tumbling frequency by these reagents; the question is, How do they act? We show here that, on the basis of capillary assays and on direct observation, uncouplers of oxidative phosphorylation and inhibitors of electron transport repel *Bacillus subtilis* and cause it to tumble temporarily, which suggests that diminution of the high energy state of the membrane or membrane potential (*4*) affects tumbling frequency but that the actual level of the high energy state itself does not govern it.

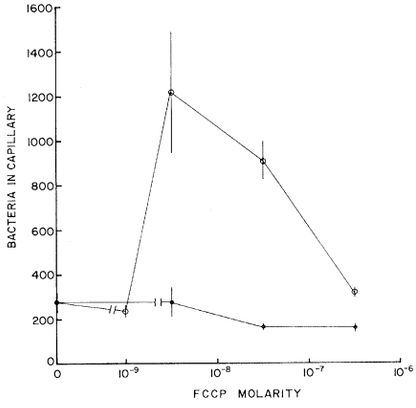

Fig. 1. Capillary assay of the repellent FCCP. Bars indicating standard error of each mean are shown. Symbols are as follows: ○, FCCP in pond and buffer in capillary; ●, FCCP in both pond and capillary.

Three related strains of *B. subtilis* were used in this study: (i) OI 1, which swims mostly, tumbles little; (ii) OI 8, which tumbles slightly more than OI 1; and (iii) OI 151, which swims little and tumbles mostly (*5*). The capillary assay, using OI 8, was performed by the "repellent in pond" method of Tso and Adler (*6*). The test substance in the bacterial suspension diffuses into a capillary containing buffer to form a gradient; bacteria flee down this gradient into the capillary. In the control experiment, the test substance is present in both capillary and pond from the beginning. If more bacteria collect in the capillary containing originally only buffer, the reagent is a repellent.