

# Minimal hydrolysis of epidermal growth factor by gastric fluid of preterm infants

J R BRITTON, C GEORGE-NASCIMENTO, J N UDALL, AND O KOLDOVSKÝ

From the University of Arizona, Department of Pediatrics, Tucson, Arizona, and Chiron Corporation, Emeryville, California, USA

**SUMMARY** Epidermal growth factor (EGF), present in high concentrations in milk, may play a role in growth of the gastrointestinal tract. Resistance to proteolytic degradation in the stomach is necessary if ingested EGF is to function within the gastrointestinal tract. Although EGF stability to low pH and proteases predicts gastric survival, the extent of digestion in the stomach remains to be defined. Consequently, we measured gastric degradation of <sup>125</sup>I-human recombinant EGF in preterm infants with an *in vitro* method in which EGF was incubated at 37°C with stomach fluid at pH 1·8, 3·2, and 5·8 followed by analysis of degradation products. As maximal acid proteolytic activity is present one hour after feeding in preterm infants, fluid was obtained at that time from 18 infants with a mean gestational age at birth of 30·4 (3·0) (SD) weeks and a postnatal age of 26·3 (12·7) days at sampling. Incubations for up to 60 minutes revealed minimal loss of trichloroacetic acid precipitable radioactivity, in contrast to the substantial hydrolysis of iodinated casein which occurred under the same conditions. Chromatography of reaction mixtures on Sephadex G-25 showed a single major peak of radioactivity which coeluted with EGF. Epidermal growth factor also retained >75% of its ability to bind to anti-EGF affinity columns and placental membrane receptors after incubation with gastric fluid. These data support the concept of substantial gastric survival of ingested EGF in a potentially biologically active form in preterm infants.

The milk of several species has growth promoting effects *in vivo* and *in vitro*.<sup>1,2</sup> The gastrointestinal tract of pigs, rabbits, dogs, and rats of milk fed newborns has been shown to have increased weight, protein, and DNA content compared with unfed or formula fed controls.<sup>3-7</sup> In tissue culture, milk is mitogenic for several cell lines, and in some cases it may replace serum in the media.<sup>8-10</sup> Among the several growth promoting activities in milk, epidermal growth factor (EGF) has emerged as one of the foremost.<sup>11,12</sup> A polypeptide with a molecular weight of about 6000 daltons,<sup>13</sup> EGF is present in high concentrations in human milk and colostrum but wide variations in individual levels exist.<sup>14-16</sup> Some<sup>14</sup> but not other<sup>15</sup> studies have found higher EGF

concentrations in colostrum than in mature milk. Although EGF is also produced in the submandibular and Brunner's glands,<sup>17-20</sup> endogenous concentrations, studied thus far only in rodents, are low during the neonatal period,<sup>17,19</sup> leading to speculation that milk may be a major source of EGF for the suckling mammal.

An increasing body of evidence suggests that EGF may be trophic for various regions of the gastrointestinal tract, including stomach, small intestine, colon, pancreas, and liver.<sup>21-33</sup> In addition, studies in rats support the concept of absorption of intact EGF from the gastrointestinal tract with delivery to peripheral tissues during the neonatal period.<sup>34-36</sup> In human infants, the role of EGF in early development remains to be defined, but it is possible that it may act within the gut or be absorbed and act at distant body sites. Promotion of growth might be especially important for premature infants, who often show

Address for correspondence: John R Britton, MD, PhD, Veterans Administration Medical Center, 1601 Perdido Street, New Orleans, LA 70146, USA.

Accepted for publication 11 August 1988.

growth delay and to which additional growth promoting activities could be valuable. In addition, EGF has been shown to be a potent inhibitor of gastric acid secretion<sup>37,38</sup> and to have gastrointestinal cytoprotective effects,<sup>39,40</sup> and it is possible that this role could also be ascribed to milk EGF. For such phenomena to occur, resistance to digestive processes within the stomach and intestines is required, at least in part: EGF must survive in either its intact form or as an active proteolytic digestion product. Although EGF resistance to low pH and a number of proteolytic enzymes *in vitro* has been shown,<sup>13</sup> the extent of digestion of dietary EGF in the human infant is unknown. Accordingly, we evaluated the gastric digestion of human EGF by measuring its digestibility by stomach fluid, as this is the first digestive secretion to which the growth factor is exposed after ingestion. Because of the potential importance of EGF for the preterm infant, gastric fluid was obtained from a group of such infants after feeding induced stimulation of gastric secretion.

## Methods

### COLLECTION OF GASTRIC FLUID

This project was approved by the Human Subjects Committee of the University of Arizona Health Sciences Center. Informed parental consent was obtained before collection of samples. Eighteen preterm infants (nine boys, nine girls) with a gestational age at birth of 30.4 (3.0) mean (SD) weeks were studied at a postnatal age of 26.3 (12.7) days. All infants were receiving intermittent orogastric feedings of either a preterm formula (15 infants) or fortified human milk (three infants). Between 0800 and 1200 hours on the morning of collection, the infants received a test feeding of 17.9 (6.4) ml/kg body weight.

Because previous studies have shown maximum gastric acid proteolytic activity at 60 minutes after a feed in premature infants,<sup>41</sup> gastric fluid was aspirated at this time into plastic containers on ice. After a 1:5 dilution with normal saline and homogenisation with a glass Teflon homogeniser, the fluid was assayed directly for proteolytic activity and EGF degradation as described. In some experiments, fluid was stored under liquid nitrogen for up to five days; such storage did not affect the rate of hydrolysis of casein nor did it alter the digestibility of EGF.

### ANALYSIS OF HYDROLYSIS OF EGF

Experiments were designed to investigate the proteolytic activity of gastric fluid toward EGF and the nature of the reaction products. The samples of gastric fluid had measured pH values between 5 and 6, in accordance with previously reported values for

gastric pH after milk feedings in the newborn.<sup>42-43</sup> To evaluate EGF degradation under these conditions, hydrolysis was measured in the presence of 0.1 M maleate buffer, pH 5.8; in addition, measurements were also performed in the presence of 0.05 N HCl, pH 1.8 (as used in the classical Anson assay for gastric peptic activity<sup>44</sup>) and with 0.1 M glycine-HCl, pH 3.2, as the latter pH approximates that of the unfed newborn stomach.<sup>45-46</sup> The rate of hydrolysis of EGF was measured at 37° in a 0.15 ml reaction mixture containing 0.05 ml of gastric fluid and 150 ng of human recombinant EGF<sup>47</sup> labelled with <sup>125</sup>I.<sup>48</sup> This EGF has been extensively characterised and is identical to gamma urogastrone.<sup>49</sup> After incubation for various time periods, the reaction was terminated by the addition of 1.0 ml 10% trichloroacetic acid (TCA) containing 0.2% bovine serum albumin and chilling on ice. Hydrolysis was determined by measuring the generation of acid soluble radioactivity as previously described.<sup>50,51</sup> In all experiments, the hydrolysis of iodinated bovine alpha casein (Sigma) to TCA-soluble material was measured as a control.<sup>50</sup> Such assays were done under conditions giving linearity with respect to amount of gastric fluid and incubation time, and results were expressed as micrograms casein hydrolysed/hour in the standard reaction mixture. For EGF, hydrolysis was expressed as the per cent of label remaining acid precipitable after a 60 minute incubation.

### ANALYSIS OF REACTION PRODUCTS

Reaction mixtures prepared as above but containing 1 ng EGF were incubated for up to one hour at 37°C and terminated by the addition of cold 0.1 M Tris-HCl, pH 8.0. This resulted in pH values above neutrality and consequent obliteration of proteolytic activity. Reaction products were analysed by three methods, each of which was chosen to assess the structural intactness of EGF by different criteria:

#### *Gel filtration*

Mixtures were chromatographed on Sephadex (Pharmacia) G-25 columns (24×1 cm) equilibrated and eluted with 0.05 M phosphate buffer, pH 7.4, containing 0.075 M NaCl. Forty 1 ml fractions were collected and counted in a gamma counter. Intact EGF typically elutes in the void volume, giving a single major peak of radioactivity (Figure). In some experiments, this peak was rechromatographed on Sephacryl S-200 (Pharmacia) using the same buffer.

#### *Affinity chromatography*

Reaction mixtures were applied to a 2×1 cm Sepharose 4B (Sigma) column to which monospecific polyclonal rabbit anti-human EGF had been complexed.<sup>54</sup> Columns were eluted with Hank's solution,

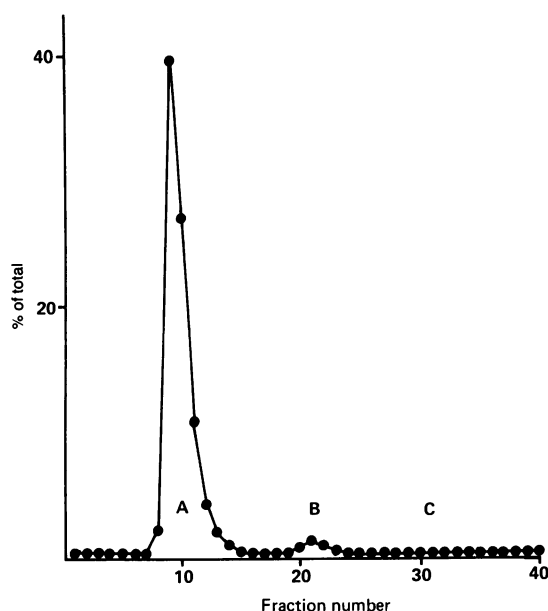


Figure Gel filtration chromatography of EGF incubated with gastric fluid. Labelled EGF was incubated with gastric fluid pooled from five infants and subjected to chromatography on Sephadex G-25 as described in Methods. (A) denotes position of intact EGF, which elutes in the void volume as determined with blue Dextran. Inorganic iodine elutes in peak (B) with the column volume, and iodotyrosines show retarded elution at (C). Identical profiles were obtained with incubations for 0 and 60 minutes at pH 5.8 and 3.2.

pH 7.4, and the percentage of retained radioactivity was determined. Typically, greater than 90% of the radioactivity in an EGF preparation was retained by the columns; boiling reaction mixtures containing labelled EGF for seven minutes resulted in reduction of binding to affinity columns to less than 2% of original values.

#### Receptor binding

Reaction mixtures were assayed for binding to human placental EGF receptors essentially as described by Hock and Hollenberg<sup>52</sup> and Hock *et al.*<sup>53</sup> Under these conditions, 33–35% of added labelled EGF binds at 25°C, and non-specific binding, measured in the presence of a 1000-fold excess of unlabelled EGF, is 5–10%.

For the latter two methods, results were expressed as a percentage of a corresponding zero time sample, prepared by incubation with boiled gastric fluid; this gave values in each assay identical to that of EGF alone.

#### STATISTICAL ANALYSIS

Data are expressed as mean (standard deviation)

(SD)) of determinations on each gastric fluid. Comparison between different groups was performed using analysis of variance and Student's unpaired *t* test, with significance accepted at  $p < 0.05$ .

#### Results

Analysis of hydrolysis of EGF by gastric fluid is shown in the Table. Incubations carried out for up to one hour revealed no increase in acid soluble radioactivity, with over 98% of the added label remaining TCA-precipitable. In contrast, iodinated bovine casein incubated under identical conditions was substantially hydrolysed to soluble material; the rate of casein degradation was significantly greater at the acid pH values than at pH 5.8, in keeping with the known pH optima of gastric proteases.<sup>44,54</sup>

Chromatographic analysis of EGF which had been exposed to human gastric fluid for one hour *in vitro* is shown in the Figure. A single major peak of radioactivity eluted in the void volume of the column (peak A), a position identical to that of intact EGF. Minimal amounts of label were present in a second peak (B) which eluted at the position of inorganic iodine and other undefined peptides<sup>34</sup> and no material was present at the position of iodotyrosines (C); there was no increase in the quantity of the iodine peak when compared with a zero time sample. To assess the possibility of aggregation or breakdown products with EGF, peak A was rechromatographed on Sephacryl S-200; a single peak was obtained which eluted in a position corresponding to intact EGF (data not shown). This material also retained 80.9% of its ability to bind to placental receptors compared to unincubated EGF. Similar results were obtained with incubations at pH 3.2 and 5.8.

The percentage of EGF which retained its ability to bind to anti-EGF affinity columns and placental

Table EGF degradation by gastric fluid

|  | pH                   |                     |                      |
|--|----------------------|---------------------|----------------------|
|  | 5.8                  | 3.2                 | 1.8                  |
| TCA precipitable material                | 99.5 (0.5)<br>[6]    | 98.0 (2.4)<br>[16]  | 99.0 (0.7)<br>[11]   |
| Antibody binding                         | 97.8 (4.2)<br>[7]    | 97.7 (9.7)<br>[14]  |                      |
| Receptor binding                         | 74.9 (18.9)<br>[6]   | 78.9 (18.1)<br>[6]  |                      |
| Hydrolysis of casein ( $\mu\text{g/h}$ ) | 1.23 (1.09)*<br>[14] | 4.77 (6.17)<br>[14] | 10.48 (9.47)<br>[14] |

Values represent the mean (SD) of determinations on the number of samples shown in square brackets. For each method, the amount of EGF detected after incubation for 60 minutes at 37°C is expressed as a percentage of that in a zero time sample. \*Different from values at lower pH,  $p < 0.05$ .

membrane receptors after incubation with gastric fluid is also shown in the Table. Binding to affinity columns was reduced by less than 3%, and greater than 75% of the ability to bind to placental EGF receptors was retained after incubation with gastric fluid.

No difference in gastric fluid EGF degradation was observed between breast fed and formula fed infants.

## Discussion

These experiments support the concept of substantial gastric survival of dietary EGF in premature infants. The inability to detect hydrolysis of EGF to acid soluble peptides suggests the absence of proteolytic cleavage of the primary structure of the peptide. Although this type of assay is generally quite sensitive when used with radioactive substrates,<sup>55</sup> it may not, however, detect the generation of peptide fragments which do not contain tyrosine, the amino acid residue which is iodinated.<sup>48</sup> Human EGF, a 53 amino acid peptide with three interconnected loops held by disulphide bonds, contains tyrosine at positions 13, 22, 29, 37, and 44,<sup>56</sup> and it is possible that fragments cleaved from the peptide between these residues or at either the carboxy or amino terminus might escape detection.

As the antisera utilised for our affinity columns was polyclonal, the retention of substantial antibody binding ability of EGF after exposure to gastric fluid similarly does not permit conclusions regarding specific regions of the molecule. The data from the affinity columns, however, do indicate substantial preservation of tertiary structure of the peptide, the loss of which, as with heat treatment, resulted in obliteration of binding to our columns. Moreover, the retention of substantial receptor binding ability also indicates some tertiary structure preservation; indeed, at least partial preservation of carboxy terminal residues is implied by our findings, since Hollenberg and Gregory<sup>57</sup> and Cohen *et al*<sup>58</sup> observed that loss of residues 49–51 results in nearly complete loss of receptor binding ability. In contrast, proteolytic removal of amino terminal residues, which may result in more limited reduction in receptor binding,<sup>59</sup> is compatible with our data, especially as such losses might also escape detection in the TCA assay. The reduction of receptor binding by approximately 25% may be caused by a minor alteration of tertiary structure by the gastric fluid.

Petrides *et al*<sup>16</sup> have observed that beta-urogastrone is the predominant form of EGF in human milk. The EGF utilised in our experiments is identical to gamma urogastrone, one of the two major EGF species present in human urine and differing from

beta-urogastrone by absence of the carboxy-terminal arginine.<sup>57,61</sup> Both beta- and gamma-urogastrone are resistant to proteases *in vitro* and have identical biological activity.<sup>57</sup> That gastric digestion of these two forms could differ in the infant is unlikely but possible.

The gastric fluid used in these assays was obtained one hour after a milk feeding, a time of maximal acid proteolytic activity.<sup>41</sup> Our control assays with casein as substrate show that the fluid has an active proteolytic capacity which is greatest at low pH. Milk feedings are known to buffer gastric acid in the infant,<sup>42–43</sup> and the resulting rise in pH may be responsible for the lower proteolysis as observed in these control assays. Nevertheless, EGF degradation was undetectable at all of the pH values tested, including 5–8, which approximates the value of the stomach pH one hour postprandially in the infant.<sup>42,43</sup>

Although we have not shown biological activity of EGF after treatment with gastric fluid, receptor binding is known in general to correlate with mitogenicity.<sup>13</sup> The gastric survival of dietary EGF in a potentially biologically active form thus has several possible implications for the preterm infant. For example, gastric acid secretion is relatively low in the premature during the first four weeks postnatally,<sup>62</sup> and we may speculate that EGF might play a role in the inhibition of such secretion.<sup>37,38</sup> Appropriate studies correlating gastric acid secretion and dietary EGF intake in premature infants remain to be performed. Although data regarding the gastric trophic effects of EGF are also lacking in man, such effects have been well documented in rodents.<sup>22,30–33</sup>

Gastric survival of EGF suggests that at least some of the active peptide present in the diet might reach the intestine. Although the extent of intestinal digestion of EGF is unknown in man, experiments in suckling rats suggest substantial survival and absorption in an intact form.<sup>34–36,63</sup> Although the extent of biological action of dietary EGF in the premature infant remains unknown, its survival in a potentially biologically active form suggests the availability for possible action within the stomach and perhaps other regions of the gastrointestinal tract. The susceptibility of dietary EGF to gastric luminal hydrolysis in term infants or in preterm infants with progressive post-natal age remains to be explored.

The authors thank M Morrill for expert technical assistance. Supported by National Institutes of Health grant AM27624 and by grants from the Nestle Research Foundation and Flinn Foundation.

## References

- 1 Koldovský O, Thornburg W. Hormones in milk. *J Pediatr Gastroenterol Nutr* 1987; 6: 172–96.



- 2 Sheard NF, Walker WA. The role of breast milk in the development of the gastrointestinal tract. *Nutr Rev* 1988; **46**: 1–8.
- 3 Widdowson EM, Colombo VE, Artavanis CA. Changes in the organs of pigs in response to feeding for the first 24 h after birth. II. The digestive system. *Biol Neonate* 1976; **28**: 272–281.
- 4 Hall RA, Widdowson EM. Response of the organs of rabbits to feeding during the first days after birth. *Biol Neonate* 1979; **35**: 131–9.
- 5 Heird WC, Schwarz SM, Hansen IH. Colostrum-induced enteric mucosal growth in beagle puppies. *Pediatr Res* 1984; **18**: 512–5.
- 6 Berseth CL, Lichtenberger LM, Morriss FH. Comparison of the gastrointestinal growth-promoting effects of rat colostrum and mature milk in newborn rats in vivo. *Am J Clin Nutr* 1983; **37**: 52–60.
- 7 Berseth CL. Breast-milk-enhanced intestinal and somatic growth in neonatal rats. *Biol Neonate* 1987; **51**: 53–9.
- 8 Klagsbrun M. Human milk stimulates DNA synthesis and cellular proliferation in cultured fibroblasts. *Cell Biol* 1978; **75**: 5057–61.
- 9 Klagsbrun M, Neumann J. The serum-free growth of Balb/c 3T3 cells in medium supplemented with bovine colostrum. *J Supramol Struct* 1979; **11**: 349–59.
- 10 Brown KD, Blakeley DM. Cell growth-promoting activity in mammary secretions of the goat, cow, and sheep. *Br Vet J* 1983; **139**: 68–78.
- 11 Carpenter G. Epidermal growth factor is a major growth-promoting agent in human milk. *Science* 1980; **210**: 198–9.
- 12 Shing YW, Klagsbrun M. Human and bovine milk contain different sets of growth factors. *Endocrinology* 1984; **115**: 273–82.
- 13 Carpenter G, Cohen S. Epidermal growth factor. *Ann Rev Biochem* 1979; **48**: 193–216.
- 14 Beardmore JM, Lewis-Jones DI, Richards RC. Urogastrone and lactose concentrations in precolostrum, colostrum, and milk. *Pediatr Res* 1983; **17**: 825–8.
- 15 Moran JR, Courtney ME, Orth DN, *et al.* Epidermal growth factor in human milk: daily production and diurnal variation during early lactation in mothers delivering at term and at premature gestation. *J Pediatr* 1983; **103**: 402–5.
- 16 Petrides PE, Hosang M, Shooter E, Esch FS, Bohlen P. Isolation and characterization of epidermal growth factor from human milk. *FEBS Lett* 1985; **187**: 89–95.
- 17 Byyny RL, Orth DN, Cohen S. Radioimmunoassay of epidermal growth factor. *Endocrinology* 1972; **90**: 1261–6.
- 18 Skov Olsen P, Nexø E. Quantitation of epidermal growth factor in the rat. Identification and partial characterization of duodenal EGF. *Scand J Gastroenterol* 1983; **18**: 771–6.
- 19 Schaudies RP, Grimes J, Davis D, Rao RK, Koldovský O. Epidermal growth factor (EGF) in rat gastrointestinal tract tissues: Effect of age and fasting. *Gastroenterology*, 1988 (In press).
- 20 Elder JB, Williams G, Lacey E, Gregory H. Cellular localization of human urogastrone/epidermal growth factor. *Nature* 1978; **271**: 466–7.
- 21 Lebenthal E, Leung Y-K. Epidermal growth factor (EGF) and the ontogeny of the gut. *J Pediatr Gastroenterol Nutr* 1987; **6**: 1–5.
- 22 Scheving LA, Yeh YC, Tsai TH, Scheving LE. Circadian phase-dependent stimulatory effects of epidermal growth factor on deoxyribonucleic acid synthesis in the tongue, esophagus, and stomach of the adult male mouse. *Endocrinology* 1979; **105**: 1475–80.
- 23 Scheving LA, Yeh YC, Tsai TH, Scheving LE. Circadian phase-dependent stimulatory effects of epidermal growth factor on deoxyribonucleic acid synthesis in the duodenum, jejunum, ileum, caecum, colon, and rectum of the adult male mouse. *Endocrinology* 1980; **106**: 1498–503.
- 24 O'Loughlin EV, Chung M, Hollenberg M, Hayden J, Zahavi I, Gall DG. Effect of epidermal growth factor on ontogeny of the gastrointestinal tract. *Am J Physiol* 1985; **249**: 674–8.
- 25 Ulshen MH, Lyn-Cook LE, Raasch RH. Effects of intraluminal epidermal growth factor on mucosal proliferation in the small intestine of adult rats. *Gastroenterology* 1986; **91**: 1134–40.
- 26 Arsenault P, Ménard D. Stimulatory effects of epidermal growth factor on deoxyribonucleic acid synthesis in the gastrointestinal tract of the suckling mouse. *Comp Biochem Physiol (B)* 1987; **86**: 123–7.
- 27 Berseth CL. Enhancement of intestinal growth in neonatal rats by epidermal growth factor in milk. *Am J Physiol* 1987; **253**: 662–5.
- 28 Pollack PF, Goda T, Colony PC, *et al.* Effects of enterally fed epidermal growth factor on the small and large intestine of the suckling rat. *Regul Pept* 1987; **17**: 121–32.
- 29 Opleta K, O'Loughlin EV, Shaffer EA, Hayden J, Hollenberg M, Gall DG. Effect of epidermal growth factor on growth and postnatal development of the rabbit liver. *Am J Physiol* 1987; **253**: 622–6.
- 30 Falconer J. Oral epidermal growth factor is trophic for the stomach in the neonatal rat. *Biol Neonate* 1987; **52**: 347–50.
- 31 Feldman EJ, Aures D, Grossman MI. Epidermal growth factor stimulates ornithine decarboxylase activity in the digestive tract of mouse. *Proc Soc Exp Biol Med* 1978; **159**: 400–2.
- 32 Johnson LR, Guthrie PD. Stimulation of rat oxyntic gland mucosal growth by epidermal growth factor. *Am J Physiol* 1980; **238**: 45–9.
- 33 Dembinski AB, Johnson LR. Effect of epidermal growth factor on the development of rat gastric mucosa. *Endocrinology* 1985; **116**: 90–4.
- 34 Thornburg W, Matrisian L, Magun B, Koldovský O. Gastrointestinal absorption of epidermal growth factor in suckling rats. *Am J Physiol* 1984; **246**: 80–5.
- 35 Thornburg W, Rao RK, Matrisian LM, Magun BE, Koldovský O. Effect of maturation on gastrointestinal absorption of epidermal growth factor in rats. *Am J Physiol* 1987; **253**: 68–71.
- 36 Popliker M, Shatz A, Avivi A, Ullrich A, Schlessinger J, Webb CE. Onset of endogenous synthesis of epidermal growth factor in neonatal mice. *Dev Biol* 1987; **119**: 38–44.
- 37 Bower JM, Camble R, Gregory H, Gerring EL, Will-

- shire IR. The inhibition of gastric acid secretion by epidermal growth factor. *Experientia* 1975; **31**: 825–6.
- 38 Elder JB, Ganguli PC, Gillespie IE, Gerring EL, Gregory H. Effect of urogastrone on gastric secretion and plasma gastrin levels in normal subjects. *Gut* 1975; **16**: 887–93.
- 39 Konturek SJ, Radecki T, Brzozowski T, *et al.* Role of endogenous prostaglandins and DNA synthesis. *Gastroenterology* 1981; **81**: 438–43.
- 40 Kirkegaard P, Skov Olsen P, Poulsen SS, Nexo E. Epidermal growth factor inhibits cysteamine-induced duodenal ulcers. *Gastroenterology* 1983; **85**: 1277–83.
- 41 Yahav J, Carrion V, Lee PC, Lebenthal E. Meal-stimulated pepsinogen secretion in premature infants. *J Pediatr* 1987; **110**: 949–51.
- 42 Mason S. Some aspects of gastric function in the newborn. *Arch Dis Child* 1962; **37**: 387–91.
- 43 Harries JT, Fraser AJ. The acidity of the gastric contents of premature babies during the first fourteen days of life. *Biol Neonate* 1968; **12**: 186–93.
- 44 Anson ML. The estimation of pepsin, trypsin, papain and cathepsin with haemoglobin. *J Gen Physiol* 1939; **22**: 79–89.
- 45 Avery GB, Randolph JG, Weaver T. Gastric acidity in the first day of life. *Pediatrics* 1966; **37**: 1005–7.
- 46 Miclat NN, Hodginson R, Marx GF. Neonatal gastric pH. *Anesth Analg* 1978; **57**: 98–101.
- 47 Urdea MS, Merryweather JP, Mullenbach GT, *et al.* Chemical synthesis of a gene for human epidermal growth factor urogastrone and its expression in yeast. *Proc Natl Acad Sci USA* 1983; **80**: 7461–5.
- 48 Greenwood FD, Hunter WM, Glover JS. The preparation of <sup>125</sup>I-labelled human growth hormone of high specific radioactivity. *Biochem J* 1963; **89**: 114–23.
- 49 George-Nascimento C, Gyenes A, Halloran SM, Merryweather J, *et al.* Characterization of recombinant human epidermal growth factor produced in yeast. *Biochemistry* 1988; **27**: 797–802.
- 50 Britton J, Koldovský O. Luminal digestion of lactoferrin in suckling and weanling rats. *Am J Physiol* 1987; **253**: 397–403.
- 51 Britton J, Koldovský O. Development of luminal protein digestion in suckling and weanling rats. *Biol Neonate* 1988; **53**: 39–46.
- 52 Hock RA, Hollenberg MD. Characterization of the receptor for epidermal growth factor-urogastrone in human placenta membranes. *J Biol Chem* 1980; **255**: 10731–6.
- 53 Hock RA, Nexo E, Hollenberg MD. Solubilization and isolation of the human placenta receptor for epidermal growth factor urogastrone. *J Biol Chem* 1980; **255**: 10737–43.
- 54 Defize J, Meuwissen SGM. Pepsinogens: An update of biochemical, physiological, and clinical aspects. *J Pediatr Gastroenterol Nutr* 1987; **6**: 493–508.
- 55 Bergmeyer HU. *Methods of enzymatic analysis*. Weinheim: Verlag Chemie, 1983: 258–70.
- 56 Gregory H, Preston BM. The primary structure of human urogastrone. *In J Pept Protein Res* 1977; **9**: 107–18.
- 57 Hollenberg MD, Gregory H. Epidermal growth factor-urogastrone: biological activity and receptor binding of derivatives. *Mol Pharmacol* 1980; **17**: 314–20.
- 58 Cohen S, Carpenter G, Lembach KJ. Interaction of epidermal growth factor (EGF) with cultured fibroblasts. *Adv Metab Disord* 1975; **8**: 265–84.
- 59 Riemen MW, Wegrzyn RJ, Baker AE, *et al.* Isolation of multiple biologically and chemically diverse species of epidermal growth factor. *Peptides* 1987; **8**: 877–85.
- 60 Heath W, Merrifield RB. A synthetic approach to structure-function relationships in the murine epidermal growth factor molecule. *Proc Natl Acad Sci USA* 1986; **83**: 6367–71.
- 61 Hirata Y, Orth DN. Epidermal growth factor (urogastrone) in human fluids: size heterogeneity. *J Clin Endocrinol Metab* 1979; **48**: 673–9.
- 62 Hyman PE, Clarke DD, Everett SL, *et al.* Gastric acid secretory function in preterm infants. *J Pediatr* 1985; **106**: 467–71.
- 63 Gonnella PA, Siminoski K, Murphy RA, Neutra MR. Transepithelial transport of epidermal growth factor by absorptive cells of suckling rat ileum. *J Clin Invest* 1987; **80**: 22–32.