Gastrin-Mediated Changes in Tyrosine Kinases in Isolated Gastric Mucosal Cells from Young and Aged Rats (43548)

ADHIP P. N. MAJUMDAR*,[1] AND MONA WAHBY†

Department of Veterans Affairs Medical Center, Allen Park, Michigan 48101 and Department of Medicine,† Division of Gastroenterology, Wayne State University School of Medicine, Detroit, Michigan 48202*

Abstract. **The present investigation examines the changes in tyrosine kinase (Tyr-k) activity and tyrosine-specific phosphorylation of membrane protein(s) in isolated gastric mucosal cells from young (3 months) and aged (22 months) Fischer-344 rats after exposure to gastrin. In mucosal cells from young rats, a 15-min time course study revealed a steady rise of Tyr-k activity for the first 2 min in response to 10^{-9} *M* gastrin (G-17 I), whereafter the enzyme activity decreased sharply. In these cells, a significant 90% ($P < 0.001$) increase in the enzyme activity was observed only after 30 sec of exposure to gastrin, with maximal stimulation (124%; $P < 0.001$) occurring after 2 min. These increases were also associated with a 2- to 3-fold rise in tyrosine-specific phosphorylation of 55-, 80-, and 100-kDa membrane proteins. In contrast, in mucosal cells from aged rats, neither Tyr-k activity nor tyrosine phosphorylation of membrane proteins was affected by gastrin with doses up to 10^{-6} *M*. Our observation of an immediate induction of Tyr-k activity and tyrosine phosphorylation of certain membrane proteins by gastrin in gastric mucosal cells from young, but not from aged, rats suggests that these events may play an important role regulating some of the physiologic actions of gastrin at different stages of life.** [P.S.E.B.M. 1993, Vol 202]

Over the past decade, there has been considerable evidence suggesting that many gastrointestinal hormones, and most notably gastrin, regulate mucosal growth in various tissues of the gastrointestinal tract, including the stomach (1, 2). However, responsiveness of the gastric mucosa to gastrin varies at different stages of life. In rats, the gastric mucosa becomes sensitive to the growth-promoting action of gastrin around the third postnatal week (3), when the parietal cells also secrete acid in response to the hormone (4). These changes have in part been attributed to the appearance of gastrin receptors in the oxyntic gland mucosa (4). In contrast, aging has been found to be associated with a loss of both acid secretory and growth-promoting actions of gastrin (5, 6). Although this could also be attributed partly to the parallel changes in gastrin receptor populations (7), the postreceptor regulatory events remain to be elucidated.

Activation of membrane tyrosine kinases (Tyr-k), which is thought to be one of the early events in the signal transduction process, is known to occur in response to a number of hormones/growth factors, including insulin, platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor (8, 9). Utilizing an organ culture system, we have recently investigated the role of Tyr-k in regulating the growth-promoting action of gastrin in the colonic mucosa of young mature rats (10). We have observed that gastrin causes a prompt rise (within 10–15 min) in Tyr-k activity and tyrosine phosphorylation of a 55- to 57-kDa membrane protein (10). These increases were found to be essential for the subsequent rise in ornithine decarboxylase activity by gastrin that occurred 4 hr later, since genistein, a specific inhibitor of Tyr-k (11), abolished the gastrin-induced stimulation of ornithine decarboxylase (10). In light of these observations, we hypothesize that Tyr-k plays a role in regulating the growth-promoting action of gastrin, and that the loss of responsiveness to this action in later life may be due

[1] To whom requests for reprints should be addressed at Education Service-14A, VA Medical Center, Allen Park, MI 48101.

Received April 14, 1992. [P.S.E.B.M. 1993, Vol 202]
Accepted October 5, 1992.

0037-9727/93/2023-0365$3.00/0

in part to the hormone's inability to activate membrane Tyr-k. To corroborate or refute this hypothesis, the present investigation examined the effect of gastrin on Tyr-k activity and tyrosine phosphorylation of membrane proteins in isolated gastric mucosal cells from young and aged rats.

Materials and Methods

Materials. [γ-^{32}P]ATP and [^{125}I]streptavidin were purchased from New England Nuclear Corp. (Boston, MA) and Amersham/Searle (Arlington Heights, IL), respectively. Collagenase (Type IA) and hyaluronidase were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin, Fraction V, was a product of Miles Laboratory (Elkhart, IN) and human synthetic gastrin G-17 I was obtained from Peninsula Laboratories (Belmont, CA). Monoclonal antiphosphotyrosine antibody was a product of Boehringer-Mannheim (Indianapolis, IN). Male Fischer-344 rats were purchased from the National Institute of Aging (Bethesda, MD) 2 months before the experiment, during which period they were fed *ad libitum* Purina rat chow.

Isolation of Gastric Mucosal Cells. Gastric mucosal cells were isolated with a slight modification (12) of the procedure described by Magous and Bali (13). Briefly, the oxyntic gland area of the stomach from overnight fasted 3-month-old (young) and 22-month-old (aged) male rats was removed and pinned to a dental wax plate containing cold HEPES-Ringer (HR) buffer (pH 7.4) (14). Mucosa was quickly stripped from the muscle layer, minced finely, and incubated for 15 min at 37°C in HR buffer containing 0.6% collagenase and 0.5% hyaluronidase. This was followed by a 10-min incubation in a fresh HR buffer containing 2 m*M* EDTA and then another 60 min in the same buffer without EDTA. At the end of the incubation period, the tissue fragments were mechanically dispersed by suction through a wide pipet orifice and then strained through a nylon mesh (180 μm). Mucosal cells were collected by centrifugation at 200*g* for 3 min and washed four times in the same buffer. A small aliquot was mixed with an equal volume of 0.4% trypan blue in HR buffer, and exclusion of the dye by the cells, as an assessment of cellular viability, was monitored. Preparations containing over 90% viable cells were used.

Incubation Conditions and Processing of Samples. In all experiments, isolated gastric mucosal cells were preincubated at 37°C for 15 min in HR buffer and then 1-ml aliquots of cell suspension were incubated again at 37°C in the absence and presence of gastrin (G-17 I), as described in the Figure Legends. The cells were recovered by centrifugation at 250*g* for 5 min, homogenized in 0.75 ml of cold homogenizing buffer (10 m*M* HEPES, pH 7.2, 150 m*M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* Na_3VO_4, 10 μg/ml of leupeptin, and 1 m*M* phenylmethylsulfonyl fluoride), and then centrifuged at 30,000*g* for 30 min to obtain the crude membrane, which was subsequently suspended in the homogenizing buffer containing 0.1% Triton X-100 and assayed for Tyr-k activity and tyrosine phosphorylation of proteins as stated below.

Tyr-k activity was determined using polymer L-Glu-L-Tyr (4:1) as substrate, as described previously (10). Briefly, the reaction mixture in a final volume of 50 μl contained 2.5 μmol of Tris·HCl, 2.5 μmol of $MgCl_2$, 0.5 nmol of orthovanadate, 0.02% Triton X-100, 3 μmol of ATP, 0.4 μCi of [γ-^{32}P]ATP, and 50 μg of Glu-Tyr polymer. The reaction at 24°C for 10 min was initiated with mucosal membrane fraction (15–20 μg of protein), terminated by applying 20 μl of the reaction mixture onto 2-cm^2 Whatman No. 3 MM filter paper, and subsequently washed and processed for measurement of radioactivity as described previously (10). The results are expressed as pmol ^{32}P incorporated/mg of protein. Protein content in all experiments was determined by the method of Bradford (15).

Autophosphorylation and identification of tyrosyl-phosphorylated proteins in the membrane fraction were carried out as described previously (10, 16), except that the reaction mixture contained 100 μg of membrane protein.

The relative concentration of phosphotyrosine membrane proteins was determined by Western blot analysis, as reported earlier (16). Briefly, an equal volume of stopping buffer (62 m*M* Tris HCl, pH 6.8, 6% sodium dodecyl sulfate, 20% glycerol, and 10% mercaptoethanol) was added to aliquots of gastric mucosal membrane preparations containing 25 μg of protein. The samples were heated at 100°C for 3 to 4 min and subsequently electrophoresed on a 7.5% polyacrylamide slab gel (0.75-mm thickness) containing 0.1% sodium dodecyl sulfate, as described previously (16). The electrophoresed proteins were then transferred to nitrocellulose membrane using LKB Trans-Blot apparatus according to the manufacturer's instructions. The membranes containing the proteins were incubated with blocking buffer (phosphate-buffered saline containing 3% bovine serum albumin, 0.25% gelatin, and 0.5% Tween 20) at 37°C for 3 hr under constant agitation, and then washed extensively with phosphate-buffered saline containing 0.1% Tween 20, followed by incubation with antiphosphotyrosine antibody (1 μg/ml) for 2 hr at 24°C. The membranes were washed again and then subjected to 1-hr incubation at 37°C with biotinylated anti-mouse IgG (1 μg/ml), and finally with [^{125}I]streptavidin for 1 hr at 37°C. After extensive washings, the membrane were exposed to X-Omat AR film. The molecular weight of the labeled bands was calculated from the marker proteins run concurrently.

Statistical Analysis. Where applicable, the results were statistically evaluated with Student's *t* test for

unpaired values, taking $P < 0.05$ as the level of significance.

Results

In the first series of experiments, the time course changes in Tyr-k activity and tyrosine-specific phosphorylation of membrane proteins were determined in gastric mucosal cells isolated from 3-month-old rats. The rationale for using 3-month-old animals in these experiments was that this age group has consistently been shown to respond to both trophic and acid secretory actions of gastrin (1, 2). In addition, gastrin has been found to stimulate protein synthesis in gastric mucosal cells isolated from 3- to 4-month-old rats (12). In the current 15-min time course study, gastrin at a concentration of 10^{-9} M produced an immediate increase in Tyr-k activity (Fig. 1). A significant 90% rise in Tyr-k activity was observed after 30 sec of exposure to gastrin, which increased further with time, attaining the maximal 124% increase over the basal level after 2 min, whereafter the enzyme activity decreased sharply (Fig. 1). To determine whether these changes in Tyr-k activity would also be accompanied by tyrosine-specific phosphorylation of certain membrane protein(s), membrane preparations were subjected to autophosphorylation reaction with [^{32}P]ATP, and the labeled proteins following immunoprecipitation with antiphosphotyrosine antibody were analyzed on sodium dodecyl sulfate-polyacrylamide gel. Results are shown in Figure 2. Whereas 1 min of gastrin exposure resulted in a 2- to 3-fold increase in tyrosine-specific phosphorylation of a membrane protein with M_r of 80,000, prolongation of the exposure period for another minute produced about 2-fold stimulation of tyrosine phosphorylation of two other membrane proteins with M_r of 55,000 and 100,000, when compared with the corresponding controls. These increases were greatly attenuated when the cells were exposed to gastrin for 5 min.

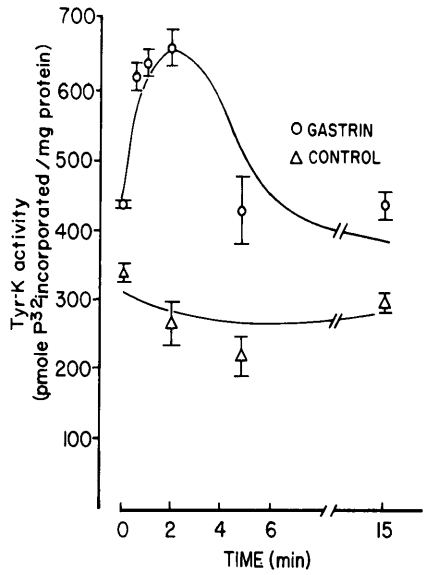

Figure 1. Time course changes in tyrosine kinase activity in isolated gastric mucosal cells from young rats in the absence (Δ) and presence (O) of 10^{-9} M gastrin. Each value represents the mean ± SE of four to five observations.

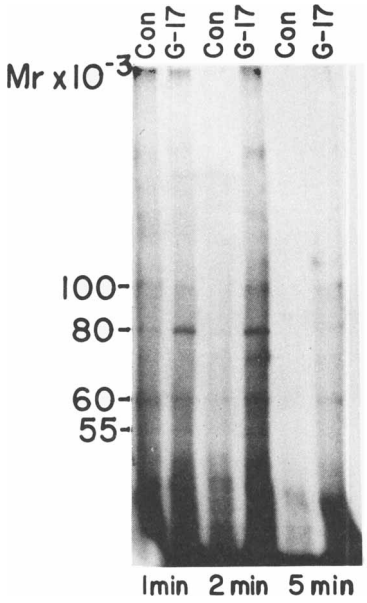

Figure 2. Effect of gastrin on tyrosine-specific phosphorylation of membrane proteins in isolated gastric mucosal cells from young rats. Gastric mucosal cells, isolated from overnight fasted 3-month-old rats, were exposed to either 10^{-9} M gastrin (G-17 I) or an equivalent volume of buffer (con) for 1, 2, or 5 min. A mucosal membrane fraction containing the same amount of protein (50 μg) was subjected to autophosphorylation reaction, followed by immunoprecipitation with antiphosphotyrosine antibody. The immunoprecipitates were electrophoresed on 7.5% polyacrylamide gel, which was subsequently processed for autoradiography.

In the next series of experiments, responsiveness of Tyr-k in isolated gastric mucosal cells from young and aged rats to gastrin was compared. Figure 3 shows the changes in Tyr-k activity in isolated gastric mucosal cells from young and aged rats after exposure to increasing concentrations of gastrin (up to 10^{-6} M) for 2 min. In mucosal cells from young rats, gastrin doses between 10^{-12} and 10^{-7} caused a steady rise in Tyr-k activity, revealing a maximal 180% increase over the basal control with a dose of 10^{-7} M. Doses beyond 10^{-7} were found to inhibit the stimulatory effect (Fig. 3). In contrast to what has been observed in young rats, none of the doses of gastrin produced any significant change in Tyr-k activity in isolated gastric mucosal cells from aged rats (Fig. 3).

To further determine whether these changes would be associated with tyrosine phosphorylation of certain membrane protein(s), isolated gastric mucosal cells from young and aged rats were exposed to 10^{-9} M gastrin for 2 min, and the membrane preparations were subjected to autophosphorylation reaction and subsequent identification of tyrosyl-phosphorylated proteins. Again, in mucosal cells from young rats, gastrin was

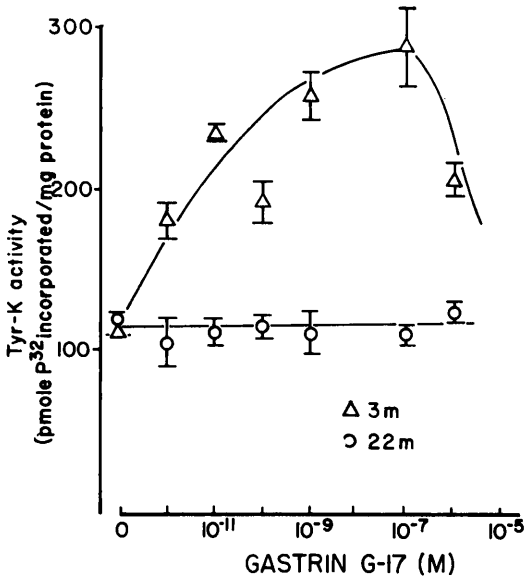

Figure 3. Effect of increasing concentration of gastrin on tyrosine kinase activity in isolated gastric mucosal cells from young (3 months) and aged (22 months) rats. Isolated gastric mucosal cells were incubated in the absence (basal) and presence of gastrin for 2 min, and Tyr-k activity in the membrane fraction was then determined. Each value represents the mean ± SE of four observations.

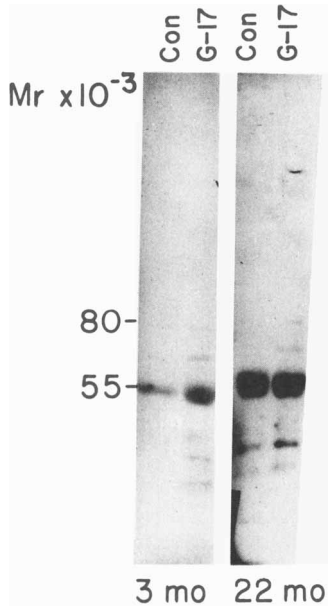

Figure 4. Effect of gastrin on tyrosine-specific phosphorylation of membrane protein in isolated gastric mucosal cells from young (3 months) and aged (22 months) rats. Gastric mucosal cells, isolated from young and aged rats, were incubated in the absence (con) and presence of 10^{-9} *M* gastrin (G-17) for 2 min. The membrane fraction was then subjected to autophosphorylation reaction and identification of tyrosine-phosphorylated proteins, as stated in Figure 2.

found to produce a marked stimulation in tyrosine phosphorylation of 55-, 80-, and 100-kDa membrane proteins (Fig. 4). In contrast, no apparent change in tyrosine phosphorylation in any of these membrane proteins was observed in response to gastrin in mucosal cells from aged rats (Fig. 4). In another set of experiments, the gastrin-mediated changes in tyrosine phosphorylation of gastric mucosal membrane proteins in young and aged rats, membrane preparations were analyzed by Western immunoblot. The results revealed an 8-fold increase in the relative concentration of 55-kDa phosphotyrosine membrane protein in mucosal cells from young rats after 2 min of exposure to gastrin when compared with the corresponding basal level (Fig. 5). Again, in mucosal cells from aged rats, gastrin produced no apparent change in the relative concentration of this phosphotyrosine membrane protein when compared with the corresponding basal level (Fig. 5). Interestingly, however, the basal concentration of 55-kDa phosphotyrosine membrane protein (data from unstimulated cells) in aged rats was found to be 10-fold higher than that in young animals (Fig. 5; control lanes).

Discussion

Although gastrin has long been known to stimulate acid secretion and growth of the gastric mucosa (17, 18), little is known about the regulatory mechanisms for these processes. Phosphorylation-dephosphorylation reactions are thought to be important mechanisms in regulating actions of many hormones. In the gastric mucosa, histamine has been shown to stimulate phosphorylation of 27- and 40-kDa proteins by cAMP-dependent protein kinase (19). In addition, 80-kDa phosphoprotein in parietal cells has also been implicated in regulating acid secretion (20). Phosphorylation of these proteins are, however, catalyzed by serine/threonine kinases.

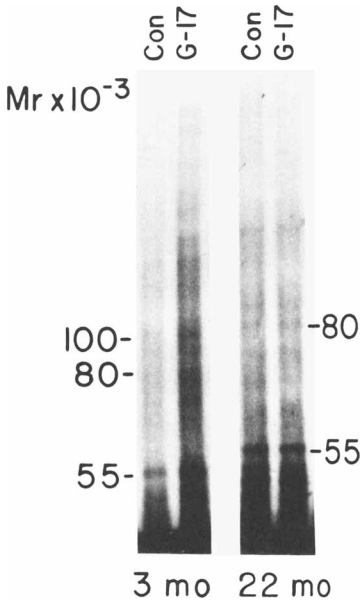

Figure 5. Western blot analysis of phosphotyrosine membrane proteins from isolated gastric mucosal cells of young (3 months) and aged (22 months) rats after incubation in the absence (con) and presence of 10^{-9} *M* gastrin (G-17) for 2 min.

Numerous evidence now indicates that tyrosine kinases, which catalyze tyrosine phosphorylation of proteins, play an important role in regulating cell proliferation (8, 21). Although little is known about specific changes in the regulatory mechanisms for mucosal cell proliferation in response to gastrointestinal hormones/peptides, recent results from this laboratory suggest a role for tyrosine kinases in these processes. At least, with epidermal growth factor, we have observed that stimulation of gastric mucosal proliferative activity in young rats and inhibition of the same in aged rats by this peptide were associated with parallel alterations in Tyr-k activity and tyrosine phosphorylation of a membrane protein with M_r of 55,000 (22). Tyrosine kinases also appear to be involved in regulating the trophic action of gastrin, since the gastrin induction of ornithine decarboxylase activity in colonic explants in organ culture could be totally abolished by the Tyr-k inhibitor, genistein (10).

Our current observation of a prompt (within a minute) rise in Tyr-k activity and tyrosine phosphorylation of certain membrane proteins in isolated gastric mucosal cells from young rats also suggests an involvement of tyrosine kinases in regulating some of the physiologic actions of gastrin. Doses of gastrin and the time required to induce maximal stimulation of Tyr-k in isolated mucosal cells were found to be far less than those observed earlier with colonic mucosal explants (10). This could be due partly to accessibility of the receptor for immediate interaction with its ligand.

Although the current data do not provide any direct evidence to implicate Tyr-k with the regulation of the trophic action of gastrin, the fact that gastrin also stimulated tyrosine phosphorylation of the 55-kDa membrane protein in mucosal cells from young rats suggests a role for Tyr-k in this process. This interpretation is supported by the observation that stimulation of gastric mucosal proliferative activity, whether it is the result of aging, injury, or being induced by administration of epidermal growth factor or bombesin to young mature rats, is associated with increased tyrosine phosphorylation of the 55-kDa membrane protein (16, 22–24). Also, in the current investigation, the basal concentration of 55-kDa phosphotyrosine protein in mucosal cells from aged rats was found to be substantially higher than in young animals (data from unstimulated cells). This could be the consequence of increased gastric mucosal proliferative activity in aged rats (16). Further support for the involvement of Tyr-k in the regulation of trophic action of gastrin comes from the observation that in isolated gastric mucosal cells from aged rats, gastrin produced no apparent change in either Tyr-k activity or tyrosine phosphorylation of the 55-kDa phosphotyrosine membrane protein. Suffice it to mention that aging has been shown to be associated with loss of responsiveness of the gastric mucosa to the trophic action of gastrin (5). Taken together, the results suggest that the age-related loss of responsiveness of the gastric mucosa to the growth-promoting action of gastrin could be due in part to the hormone's inability to activate Tyr-k and tyrosine phosphorylation of certain membrane protein(s), particularly the 55-kDa phosphotyrosine membrane protein.

The authors wish to thank Judith Leslie for typing the manuscript. The project was supported by grants to A. M. from the National Institutes of Health/National Institute of Aging (AG08438) and the Veterans Administration Medical Research Service.

1. Johnson LR. Regulation of gastrointestinal growth. In: Johnson LR, Ed. Physiology of the Gastrointestinal Tract. New York: Raven Press, pp301–333, 1987.
2. Majumdar APN. Growth and maturation of the gastric mucosa. In: Morisset J, Solomon TE, Eds. Growth of the Gastrointestinal Tract: Gastrointestinal Hormones and Growth Factors. Boca Raton, FL: CRC Press, pp119–130, 1991.
3. Majumdar APN, Johnson LR. Gastric mucosal cell proliferation during development and effects of pentagastrin. Am J Physiol **242**:G135–G139, 1982.
4. Takeuchi K, Peitsch W, Johnson LR. Mucosal gastrin receptor. V. Development in newborn rats. Am J Physiol **240**:G163–G169, 1987.
5. Majumdar APN, Edgerton EA, Dayal Y, Murthy SNS. Gastrin: Levels and trophic action during advancing age. Am J Physiol **254**:G538–G542, 1988.
6. Maitra RS, Edgerton EA, Majumdar APN. Gastric secretion during aging in pyloric-ligated rats and effects of pentagastrin. Exp Gerontol **23**:463–472, 1988.
7. Singh P, Rae-Venter B, Towensend CM Jr, Thompson J. Gastrin receptor in normal and malignant gastrointestinal mucosa: Age-associated changes. Am J Physiol **249**:G761–G769, 1985.
8. Yarden Y, Ullrich A. Growth factor receptor tyrosine kinases. Annu Rev Biochem **57**:443–478, 1988.
9. Blackshear PR. Converging and diverging pathways in polypeptide hormone action. Clin Res **37**:554–563, 1989.
10. Majumdar APN. Role of tyrosine kinases in gastrin induction of ornithine decarboxylase in colonic mucosa. Am J Physiol **259**:G626–G630, 1990.
11. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukam Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem **262**:5592–5595, 1987.
12. Majumdar APN, Edgerton EA. Gastrin induction of protein synthesis in isolated gastric mucosal cells. Proc Soc Exp Biol Med **185**:396–402, 1987.
13. Magous R, Bali JP. Evidence that proglumide and enzotript antagonize secretagogue stimulation of isolated gastric parietal cells. Regul Pept **7**:233–241, 1983.
14. Williams JA, Korc M, Dormer RL. Action of secretagogues on a new preparation of functionally intact, isolated pancreatic acini. Am J Physiol **235**:E517–E524, 1978.
15. Bradford MA. A rapid and sensitive method for the determination of microgram quantities of protein utilizing the principles of dye binding. Anal Biochem **72**:248–254, 1976.
16. Majumdar APN, Jasti S, Hatfield JS, Tureaud J, Fligiel SEG. Morphological and biochemical changes in gastric mucosa of aged rats. Dig Dis Sci **35**:1364–1370, 1990.

17. Walsh JH, Grossman MI. Gastrin. N Engl J Med **292**:1324–1334, 1975.
18. Grossman MI. Gastrin and its activities. Nature **228**:1147–1150, 1970.
19. Chew CS, Brown MR. Histamine increases phosphorylation of 27- and 40-kDa parietal cell proteins. Am J Physiol **253**:G823–G829, 1987.
20. Hanzel DK, Urushidani T, Usinger WR, Smolka A, Forte JG. Immunological localization of an 80-kDa phosphoprotein to the apical membrane of gastric parietal cells. Am J Physiol **256**:G1082–G1089, 1989.
21. Hunter T, Cooper JA. Protein tyrosine kinases. Annu Rev Biochem **57**:443–487, 1988.
22. Majumdar APN, Moshier JA, Arlow FL, Luk GD. Biochemical changes in the gastric mucosa after injury in young and aged rats. Biochim Biophys Acta **992**:35–40, 1989.
23. Majumdar APN, Arlow FL. Aging: Altered responsiveness of gastric mucosa to epidermal growth factor. Am J Physiol **257**:G554–G560, 1989.
24. Tureaud J, Majumdar APN. Aging: Diminished responsiveness of gastric mucosal ornithine decarboxylase (ODC) to bombesin *in vitro*. Gastroenterology **100**:A551, 1991.