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Gastric antisecretory activity of cycloheximide due to inhibition of protein synthesis

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Treatment of rats with cycloheximide 1 h before carbachol dose-dependently reduced the secretagogue-stimulated gastric acid secretion in pylorus ligated rats, and partially blocked carbachol- or histamine-induced activation of rat gastric $(H^+ + K^+)$-ATPase which includes translocation of reserve intracellular $(H^+ + K^+)$-ATPase into the apical membrane of the parietal cells and induction of a KCl pathway. Time-course studies showed that the drug was effective only when administered at least 30 min before the secretagogues. Puromycin showed the same effect as cycloheximide. Pulse labelling studies with [^{35}S]methionine led to identification of two most actively synthesized polypeptides in rat gastric mucosa; the proteins of 38 000 and 14 000 molecular weight. The larger polypeptide was identified as rat pepsinogen. The identity of the smaller protein is not known yet. We suggest that synthesis of nascent polypeptide(s) is required for certain steps of the acid secretory process leading to the activation of the acid pump.

Introduction

Acid secretion in gastric parietal cells is a complex process which is initiated by binding of secretagogues to their receptors at the serosal membrane and is concluded by activation of the acid pump, $(H^+ + K^+)$-ATPase, in the luminal membrane [1–3]. Propagation of the transcellular signal involves second messengers (cAMP and Ca^{2+}, so far), protein kinases, cytoskeleton [1–4] and perhaps many unknown cellular reactions. In this study we have observed that cycloheximide, an inhibitor of protein synthesis, blocked acid secretion stimulated by carbachol in rats, if the animals were treated with the drug 30 min prior to the secretagogue. The pretreatment may interfere with carbachol- or histamine-induced activation of $(H^+ + K^+)$-ATPase which includes insertion of the intracellular tubulovesicles containing $(H^+ + K^+)$-ATPase into the apical membranes and induction of a KCl pathway [5–8]. Since puromycin, another inhibitor of protein synthesis [9], produced similar results, it appears that synthesis of nascent polypeptide(s) is involved in stimulation of the acid secretory process in the parietal cells.

Materials and Methods

Male Sprague-Dawley rats weighing about 230 g were fasted overnight. Cycloheximide (1 mg/kg), cimetidine (200 mg/kg), U-17701, 4-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-fluoroethyl]-2,6-piperi-

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dindione, (2 mg/kg) and actinomycin D (2 mg/kg) were injected intraperitoneally. Puromycin (60 mg/kg) was administered intraperitoneally three times with a 30 min interval between the injections [9]. Carbachol (500 μg/kg) or two doses of histamine (30 mg/kg) with a 30 min interval between them were injected subcutaneously. Pretreatment of the rats with cycloheximide, cimetidine, U-17701 or actinomycin D was carried out usually 1 h before the secretagogues, unless specified otherwise. In case of puromycin, the third injection of the drug was followed immediately with carbachol. In those experiments designed to study protein synthesis, [^{35}S]methionine at the dose 25 to 100 μCi/rat was injected intraperitoneally 30 min after injection of cycloheximide, unless specified otherwise. The animals were killed by cervical dislocation 30 min after the second histamine injection or 1 h after the other final treatments.

Typically, scraped mucosal tissues from ten stomachs were suspended in 40 ml of ice-cold Buffer 1 containing 250 mM sucrose, 2 mM $MgCl_2$, 1 mM ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid and 2 mM Hepes-Tris pH 7.4). The gastric microsomes were prepared and resolved into the light and the heavy fractions using isotonic ^2H_2O gradient centrifugation as described previously [8]. The light microsomes have been shown to be enriched with the intracellular tubulovesicles containing ($H^+ + K^+$)-ATPase and the heavy microsomes to include the canalicular-derived membrane vesicles from the parietal cells [8]. For further purification, the heavy microsomes were resuspended in a medium made of four parts of 240 mM sucrose/10 mM Hepes buffer (pH 7.4) and one part of sucrose iso-osmotic Percoll stock and centrifuged at 18000 × *g* for 30 min in a 60 Ti rotor. The mixture was fractionated by piercing the bottom of centrifuge tube and the membranous materials were separated from Percoll as described elsewhere [10].

Assays. ($H^+ + K^+$)-ATPase activity in various gastric microsomes was estimated by measuring ouabain-insensitive K^+-dependent ATP hydrolysis as described before [8]. Intravesicular H^+-accumulation was studied by measuring amino[^{14}C]pyrine uptake or fluorescence quenching of acridine orange (1 μM) in the medium containing 40 mM KCl, 180 mM sucrose, 10 mM Pipes-Tris, 0.5 mM Mg^{2+}-ATP in the absence of valinomycin [8]. In some experiments where valinomycin (4 μg/ml) was included, the concentration of KCl was raised to 150 mM. Pepsinogen activity was determined by the hemoglobin method [11]. Protein was determined by the method of Lowry et al. [12] using bovine serum albumin as a standard. Acid secretion in vivo was measured in pylorus-ligated rats as described earlier [13]. Briefly, the fasted rats were injected with cycloheximide at doses ranging from 0.1 to 5 mg/kg. One hour later the pylorus was ligated under anesthesia. The rats were injected immediately with carbachol and were killed 2 h after pylorus ligation. The acid content of gastric juice was calculated from the total volume and acidity determined with an automatic titrator (Zymark Robotics). Each group consisted of six to ten rats.

Gel electrophoresis and autoradiograms. Polyacrylamide slab gel (10%) containing 0.09% bisacrylamide and 0.1% sodium dodecyl sulfate were prepared and electrophoresis was carried out using Laemmli's discontinuous buffer system [14]. The gels were stained with Coomassie blue and soaked in 'Enlightening' medium (New England Nuclear), dried and exposed to Kodak AR 'X-Omat' film at −70°C.

Purification of 38 K polypeptide: The suspensions of the heavy microsomes (approx. 10 mg protein/ml) were mixed with 9 vols. of 5 mM EDTA/2 mM Tris-HCl (pH 8.0) and incubated for 30 min at 23°C. The supernatant obtained after centrifugation of the mixture at 40 K for 30 min was concentrated by freeze-drying and applied to a column (1.8 × 30 cm) of Sephadex G-200 equilibrated in the Tris-EDTA buffer. The column was eluted with the Tris-EDTA buffer. The fractions enriched with 38 K polypeptide were pooled and mixed with a batch of DEAE-Sepharose resin equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The resin were extracted successively with 0.4 M, 1 M and 2 M NaCl. Supernatants of each extraction were dialyzed against H_2O and concentrated by freeze-drying. Pepsinogen from rat gastric mucosa was prepared using ammonium sulfate precipitation as described elsewhere [14].

Materials. L-[^{35}S]Methionine (1058 Ci/mmol) was purchased from New England Nuclear.

Cycloheximide, actinomycin D, puromycin, carbachol and histamine were obtained from Sigma. U-17701, 4[2-(3,5-dimethyl-2-oxocyclohexyl)-2-fluoroethyl]-2,6-piperidindione, was prepared in the Upjohn Company. Hog pepsin was purchased from Worthington.

Results

Administration of cycloheximide one hour prior to carbachol inhibited the secretagogue-induced gastric acid output in pylorus-ligated rats with an ED_{50} value of 0.3 mg/kg (Fig. 1). Further studies have shown that the pretreatment partially blocked the secretagogue-mediated translocation of gastric $(H^+ + K^+)$-ATPase. For example, during the resting state (cimetidine-treated) more than 75% of the microsomal $(H^+ + K^+)$-ATPase activity was associated with the light microsomes, whereas with carbachol treatment nearly 70% of the microsomal ATPase activity was located in the heavy microsomes (Table I). This marked change in the distribution of microsomal $(H^+ + K^+)$-ATPase has been interpreted as the secretagogue-induced insertion of reserve $(H^+ + K^+)$-ATPase in light intracellular tubulovesicles into the heavy apical membranes of the parietal cells [8]. Pretreatment of the rats with cycloheximide partially blocked the carbachol-induced translocation of the ATPase activity from the light to the heavy microsomes (Table I). We have also observed that as the animals were stimulated with carbachol, a lesser fraction of total $(H^+ + K^+)$-ATPase activity in the homogenates was recovered in the post-mitochondrial supernatant than when the animals were in the resting state (Table I). It appears that the heavy apical membranes were less readily released from the cells as microsomal fragments during homogenization than the light intracellular tubulovesicles containing $(H^+ + K^+)$-ATPase, since rehomogenization of the premicrosomal particulate fraction (enriched with nuclei and mitochondria)

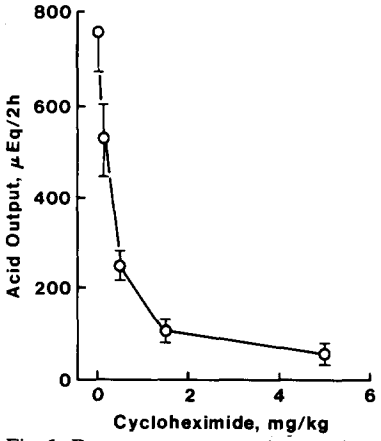


Fig. 1. Dose-response curve for gastric antisecretory activity of cycloheximide. The rats fasted for 24 h were treated with an indicated intraperitoneal dose of cycloheximide, 1 h later were pylorus-ligated and stimulated with carbachol. Each group consisted of six rats (except ten rats in the controls), and the animals were killed 2 h after carbachol treatment.

TABLE I

DISTRIBUTION OF $(H^+ + K^+)$-ATPase ACTIVITY IN THE HEAVY AND THE LIGHT MICROSOMES FROM THE RATS TREATED WITH CARBACHOL OR CYCLOHEXIMIDE FOLLOWED WITH CARBACHOL 1 h LATER, OR CIMETIDINE

The heavy and the light microsomes were obtained from ^2H_2O gradient centrifugation of post-mitochondrial supernatant as described elsewhere [8]. The data represent the mean of at least three separate experiments and standard deviation.

Treatment	Total $(H^+ + K^+)$-ATPase activity (μmol P_i/h)			
	homogenates	microsomes and soluble (post-mitochondrial supernatant)	microsomes	
			Heavy	Light
Carbachol	4667 ± 263	2546 ± 176	1542 ± 49	685 ± 63
Cycloheximide + carbachol (1 h)	4885 ± 122	3228 ± 60	1173 ± 75 *	1385 ± 85 *
Cimetidine	5509 ± 627	4667 ± 630	703 ± 75 *	2178 ± 105 *

* Value differs significantly from the carbachol-treated ($P < 0.01$).

TABLE II

COMPARISON OF SPECIFIC ACTIVITY OF $(H^+ + K^+)$-ATPase IN THE HEAVY AND THE LIGHT MICROSOMES FROM THE RATS TREATED WITH INHIBITORS OF PROTEIN SYNTHESIS AND SECRETAGOGUES, ALONE OR IN COMBINATION

The heavy and the light microsomes were isolated using ^2H_2O gradient centrifugation as described in Materials and Methods. $(H^+ + K^+)$-ATPase activity represents K^+-dependent ATP hydrolysis. When the results were available from three or more separate experiments, their mean and standard deviation are presented. In some cases the data represent the mean of two separate experiments, and experimental errors were less than 15%.

Treatment	Pretreatment time [a]	Specific activity of $(H^+ + K^+)$-ATPase (μmol P_i/h per mg protein)	
		heavy microsomes	light microsomes
Resting [b]		20.4 ± 4.8	45.7 ± 8.0
Cycloheximide [c]		22.4 ± 1.5	50.3 ± 2.8
Carbachol [d]		21.9 ± 3.4	18.4 ± 1.5
Histamine [e]		19.7	20.7
Carbachol + cycloheximide [f]	40 min	19.5 ± 1.6	18.4 ± 1.5
Cycloheximide + carbachol	0 min	23.2	18.0
	30 min	23.4	31.3
	1 h	23.7 ± 0.27	36.3 ± 4.3
	3 h	19.9 ± 0.4	34.9 ± 1.7
Cycloheximide + histamine	1 h	19.8	30.4
Puromycin [g] + carbachol	1 h	22.3	41.7
Cimetidine + carbachol	1 h	23.5	37.8

[a] Time intervals allowed after cycloheximide pretreatment until carbachol or histamine treatment.
[b] Treated with cimetidine (200 mg/kg) 1 h before killing.
[c] Treated with cycloheximide (1 mg/kg) 1 h or 3 h before killing.
[d] Treated with carbachol (500 μg/kg) 1 h before killing.
[e] Treated with histamine (30 mg/kg) 0.5 and 1 h before killing.
[f] Treated with carbachol at 0 and with cycloheximide at 40 min.
[g] Treated with 15 mg of puromycin at 0, 30 and 60 min and with carbachol at 60 min.

yielded mostly the heavy microsomes containing $(H^+ + K^+)$-ATPase [8].

Table II shows that treatment with carbachol or histamine markedly reduced the specific activity of $(H^+ + K^+)$-ATPase in the light microsomes, but with no significant effect on that of the heavy microsomes. These observations are consistent with our earlier proposal that the secretagogue treatment led to a marked increase in the overall content of $(H^+ + K^+)$-ATPase activity in the heavy apical membranes of the parietal cells by the increase in their population, probably through expansion of its surface area, but not by the increase in their specific activity [8]. Again, pretreatment with cycloheximide 1 h prior to carbachol partially blocked the carbachol or histamine-induced reduction of the ATPase activity of the light microsomes without noticeable effect on that of the heavy microsomes (Table II). These data demonstrated that the changes in the specific activity of the light microsomes correlates well with the movement of $(H^+ + K^+)$-ATPase in the parietal cell.

Thus, time-course studies showed that cycloheximide was only effective in blocking the carbachol-induced translocation of $(H^+ + K^+)$-ATPase when administered at least 30 min before the secretagogue (Table II). When given at the same time or 40 min after carbachol, cycloheximide did not interfere with the secretagogue effect, although we allowed a time period of 1 or 1.5 h between the cycloheximide treatment and the killing of the animal (Table II). It should be noted that cycloheximide within 30 min inhibited gastric mucosal protein synthesis by 80% as measured by [^{35}S]methionine incorporation into trichloroacetic

TABLE III

EFFECT OF CYCLOHEXIMIDE ON GASTRIC PROTEIN SYNTHESIS AS MEASURED WITH [^{35}S]METHIONINE INCORPORATION

The rats were treated with cycloheximide (1 mg/kg) at 30 min, 1 h or 2 h before killing except the control group, that received saline. When indicated, the rats were treated with carbachol (500 μg/kg) and [^{35}S]methionine (30 μCi per rat) 1 h and 30 min before killing, respectivley. In some experiments, carbachol treatment was omitted. The gastric mucosal tissues obtained by scraping with a glass slide was sonicated for 2 min. The ^{35}S-radioactivity was measured using a scintillation cocktail, Dimilume (Packard) in the tissue pellets obtained with 10% trichloroacetic acid treatment. The tissue was solubilized with Soluene 480.

Time after cycloheximide	[^{35}S]Methionine incorporation	
	cpm/mg protein	%
Cycloheximide and carbachol-treated		
Control	4090 ± 200	100
30 min	840 ± 42	20.5
1 h	864 ± 50	21.1
2 h	1055 ± 40	25.8
Cycloheximide alone		
Control	3040 ± 45	
30 min	801 ± 30	
1 h	753 ± 32	

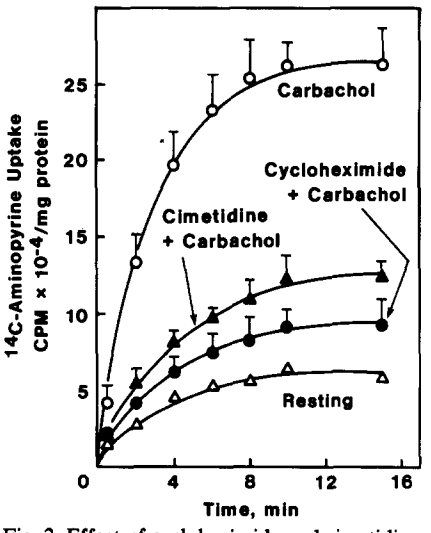


Fig. 2. Effect of cycloheximide and cimetidine on H^+-transport activity as measured with amino[^{14}C]pyrine uptake in rat gastric heavy microsomes. The heavy microsomes were prepared from rats fasted overnight (△), treated with carbachol (○), or pretreated with cycloheximide (●) or cimetidine (▲) 1 h before carbachol. The transport activity was measured in the medium containing 40 mM KCl, 180 mM sucrose, 0.5 mM Mg^{2+}-ATP, 3 μM amino[^{14}C]pyrine, 10 mM Pipes-Tris, (pH 7.0) without valinomycin at 24°C. Each point with a bar represents the mean and standard deviation of measurements from three separate experiments, each consisting of duplicate determinations.

acid-precipitable polypeptides (Table III).

These results suggest that the gastric antisecretory activity of cycloheximide could not be ascribed to general poisoning of the parietal cells, but rather to its inhibition of synthesis of a particular polypeptide(s) required for a critical step prior to activation of $(H^+ + K^+)$-ATPase. As further shown in Table II, puromycin which is another inhibitor of protein synthesis structurally quite different from cycloheximide also blocked the carbachol-induced activation of (H^+K^+)-ATPase (that is, no decrease in the ATPase activity in the light microsomes with carbachol treatment), whereas actinomycin D and a structural analog of cycloheximide, U-17701, had no effect (data not shown). Cimetidine, a H_2 receptor antagonist, also blocked the carbachol effect like the inhibitors of protein synthesis.

Fig. 2 shows that pretreatment with cycloheximide markedly inhibited carbachol-induced H^+-accumulation in the heavy microsomes as measured with amino[^{14}C]pyrine uptake in the absence of valinomycin. Similar inhibitory effects were observed with puromycin (data not shown) as well as with cimetidine. As H^+-accumulation was monitored by fluorescence decrease in acridine orange (Table IV), cycloheximide pretreatment also reduced the carbachol-induced rate of the acridine orange signal by nearly 50% in the absence, but not in the presence, of valinomycin. This observation indicates that cycloheximide partially blocked the induction of a KCl pathway in the heavy apical membrane of the parietal cells. We have further resolved the heavy microsomes using Percoll gradient centrifugation (Fig. 3). It has been found that the top two lightest subfractions were most enriched with $(H^+ + K^+)$-ATPase activity and were largely free of 38 kDa polypeptide (representing pepsinogen, see below). Again, the Percoll-purified heavy microsomes from the rats pretreated with cycloheximide 1 h prior to carbachol showed nearly 40% reduction in the rate of acridine orange fluorescence quenching as com-

TABLE IV

COMPARISON OF THE RATES FOR INTRAVESICULAR ACIDIFICATION IN THE HEAVY MICROSOMES IN THE PRESENCE OR ABSENCE OF VALINOMYCIN

The heavy or the Percoll-purified heavy microsomes (the top two fractions, see Fig. 3) from the rats treated with carbachol or with cycloheximide followed by carbachol 1 h later were suspended (approx. 100 mg) in 1 ml of a solution containing 40 mM KCl, 1 μM acridine orange and 10 mM Pipes/Tris (pH 7.0) or a solution containing 150 mM KCl, 1 μM acridine orange, 10 mM Pipes-Tris (pH 7.0) and valinomycin (4 μg/ml). The reaction was immediately initiated by adding Mg^{2+}-ATP to a final concentration of 500 μM at 23° C. The degree of H^+ accumulation was estimated by the initial slope of acridine orange fluorescence quenching.

	Initial rate of acridine orange fluorescence decrease (% change per 45 s per 100 μg protein)	
	40 mM KCl	150 mM KCl + valinomycin
Heavy microsomes		
Carbachol	9.4 ± 0.7	12.6 ± 0.9
Cycloheximide + carbachol	5.1 ± 0.8	12.2 ± 0.2
Percoll-purified heavy microsomes		
Carbachol	30.5 ± 5.2	31.6 ± 4.5
Cycloheximide + carbachol	19.0 ± 2.8	31.0 ± 4.6

pared to those from the animals treated with carbachol alone, but only in the absence of valinomycin (Table IV). The $(H^+ + K^+)$-ATPase activity was 56.7 and 63.3 μmol P_i/h per mg

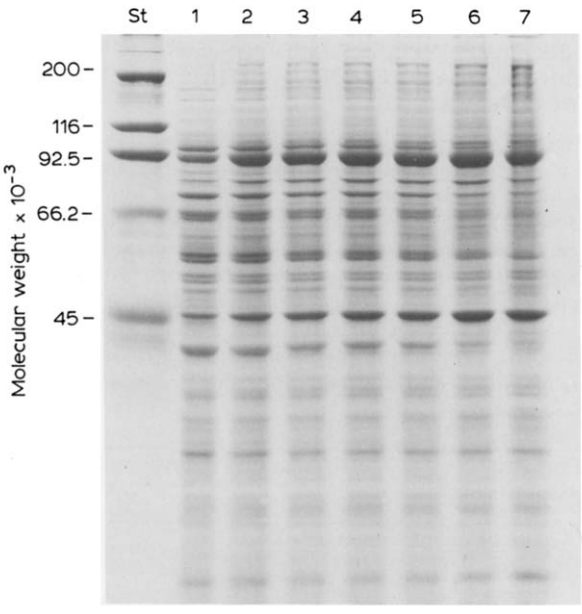


Fig. 3. SDS-polyacrylamide gel electrophoresis patterns of Percoll gradient-resolved heavy microsomes from the carbachol-treated rat. The heavy microsomes were mixed with isotonic sucrose-Percoll to a final Percoll concentration of 20% and centrifuged at 18000 × *g* for 30 min in a 60 Ti rotor. Subfractions of 3.7 ml were obtained by piercing the bottom of the tube. Lane 1 represents the heaviest subfraction and Lane 7 the top lightest fraction. About 20 μg of membrane protein were applied to 10% SDS slab gel. The gel was stained with Coomassie blue. Molecular weight standards are: myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin and ovalbumin from Bio-Rad.

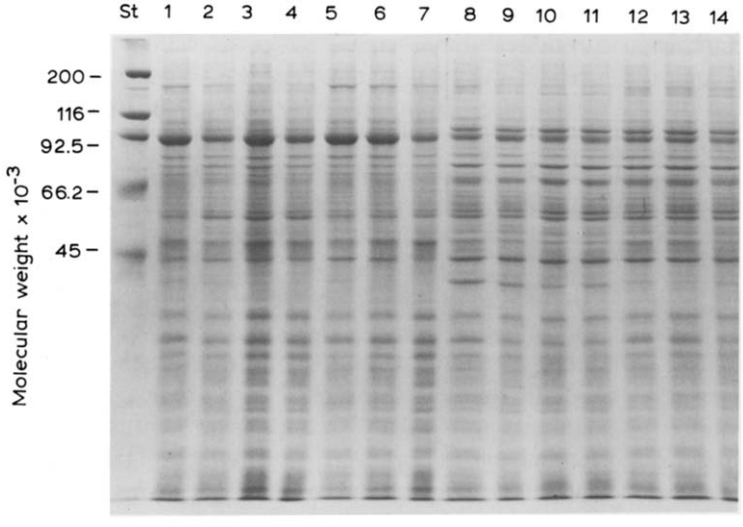


Fig. 4. Effect of cycloheximide treatment on SDS-polyacrylamide gel electrophoresis patterns of the light and the heavy microsomes from the rat gastric mucosa. The light (Lanes 1–7) and the heavy microsomes (Lanes 8–14) were prepared from the resting (lanes 1, 3, 5 and Lanes 8, 10, 12) and the carbachol-stimulated rats (Lanes 2, 4, 6, 7 and Lanes 9, 11, 13, 14) without (Lanes 1, 2 and Lanes 8, 9) or with cycloheximide treatment 1 h (Lanes 3, 4 and Lanes 10, 11), 3 h (Lanes 5, 6 and Lanes 12, 13) or 24 h (Lanes 7 and 14) before carbachol for the stimulated animals or before killing for the resting animals. 15 μg of membrane proteins were applied for electrophoresis and the gel was stained with Coomassie blue. Molecular weight standards are described in the legend of Fig. 3.

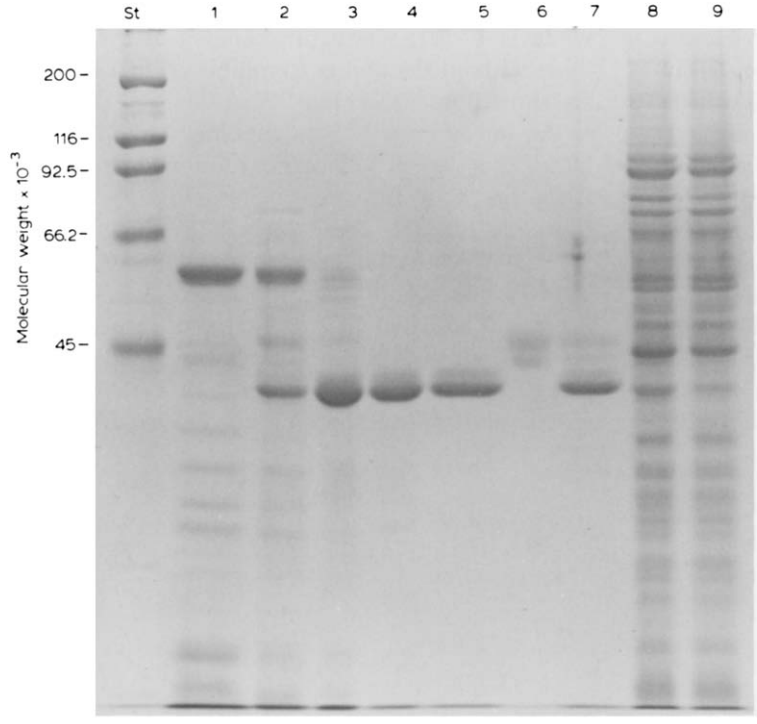
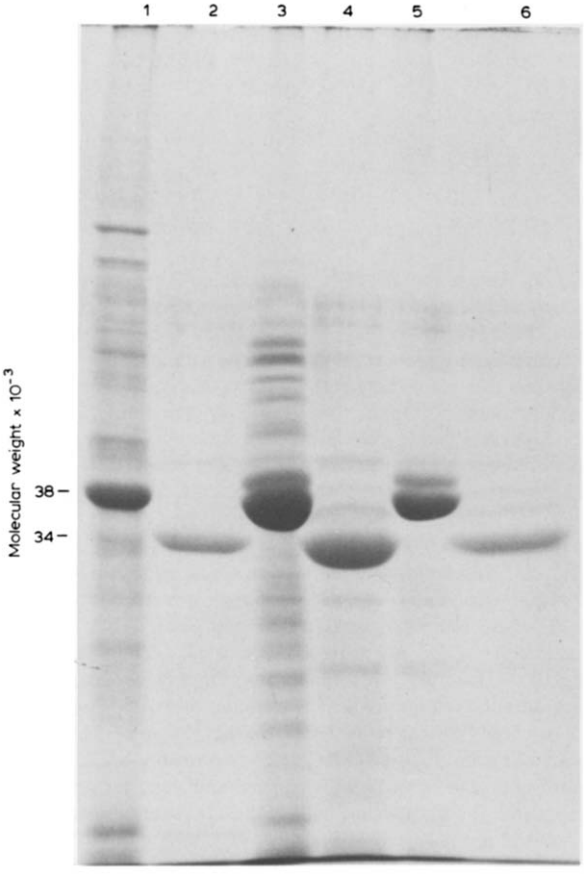


Fig. 5. Purification of the 38 kDa polypeptide from rat heavy gastric microsomes. The fraction enriched with the 38 kDa polypeptide from Sephadex G-200 chromatography of the Tris (2 mM)-EDTA (5 mM) extract of the heavy microsomes was mixed with DEAE-Sepharose resin. The supernatant which represents polypeptides unbound to the DEAE resin (Lane 1); 0.4 M NaCl extract (Lane 2), 1 M NaCl extract (Lane 3), 2 M NaCl extract (Lane 4 and 5) of the DEAE resin; commercial hog pepsin from Worthington (lane 6) with the polypeptides from Lane 5 (Lane 7) and the rat gastric heavy microsome from the resting (Lane 8) and the cycloheximide-treated rats (Lane 9). About 30 μg of protein were applied. Molecular weight standards (St) were the same as described in legend of Fig. 3.



protein for the cycloheximide-carbachol and the carbachol-treated, respectively, in these particular membrane fractions.

Cycloheximide treatment caused no noticeable effect on the polypeptide patterns of the light microsomes, but brought about one prominent change on those of the heavy microsomes, namely the reduction of the polypeptide of 38 000 (Fig. 4). The 38 kDa polypeptide was completely extracted from the heavy microsomes with 2 mM Tris/5 mM EDTA buffer (pH 8.0), and was purified through Sephadex G-200 chromatography of the extract, and batch-wise separation using DEAE-Sepharose resin (Fig. 5). The polypeptide of 38 000 which was released with 2 M NaCl from DEAE resin digested hemoglobin only under acidic con-

Fig. 6. SDS-polyacrylamide gel electrophoresis patterns showing pepsinogen and its conversion to pepsin. Lanes 1, 3 and 5 represent the 38 kDa polypeptide (pepsinogen) prepared through ammonium sulfate precipitation (Lane 1) and 2 M NaCl extract from the DEAE resin absorbed with the Tris-EDTA extracts of the havy microsomes (Lanes 3 and 5 corresponding to Lanes 4 and 5 in Fig. 5). Lanes 2, 4 and 6 represent 0.1 M HCl-treated materials of Lanes 1, 3 and 5, respectively. About 30 μg of protein were applied.

ditions with the proteolytic specific activity almost equivalent to a commercially available hog pepsin (Worthington, apparent mol. wt. 45 000). Furthermore, the rat pepsinogen we have purified through the conventional ammonium sulfate precipitation [15] comigrated with the purified polypeptide of 38 000. Both of these were converted to a polypeptide of 34 000 upon incubation in 0.06 M HCl, the likely conversion to pepsin (Fig. 6). These data led us to assign the 38 kDa polypeptide as membrane-bound rat pepsinogen. Puromycin treatment also caused disappearance of membrane-bound pepsinogen from the heavy microsomes while U 17 701, a structural analog of cycloheximide without the ability to inhibit protein synthesis, showed no effect (Fig. 7).

An injection of [^{35}S]methionine at the dose of 50 μCi per rat led to incorporation of the radioactivity primarily into pepsinogen (Fig. 8). Cycloheximide blocked about 50% of [^{35}S]methionine associated with the microsomes was 5 600 and 10 800 cpm/mg protein for the cycloheximide-carbachol and the carbachol-treated, respectively. As the dose of [^{35}S]methionine was raised to 100 μCi per rat, another polypeptide of 14 000 in the gastric mucosal microsomes was labeled from the

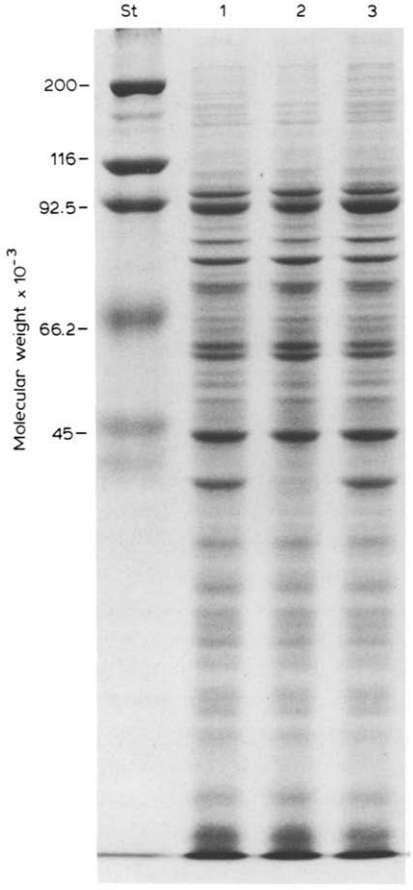


Fig. 7. Effect of puromycin and U-17 701 treatment on SDS-polyacrylamide gel electrophoresis patterns of the rat gastric heavy microsomes. The heavy microsomes were prepared from the untreated resting (Lane 1), the puromycin-treated (Lane 2) or the U-17 701-treated rats (Lane 3). Other experimental details were the same as described under Materials and Methods.

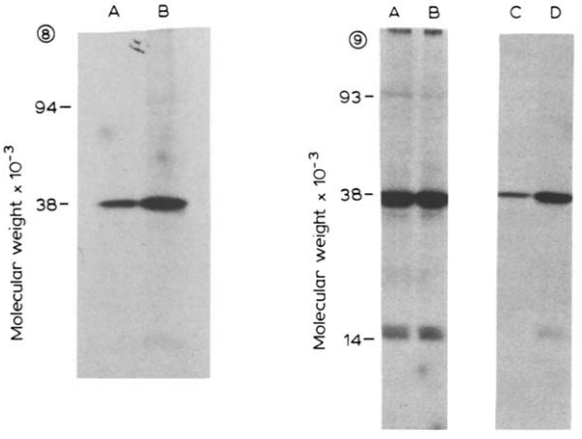


Fig. 8. Autoradiogram of [^{35}S]methionine labeled rat gastric heavy microsomes. The heavy microsomes were prepared from rats successively treated with cycloheximide, 50 μCi of [^{35}S]methionine and carbachol (Lane A) with a 30 min interval between the treatments or from those treated equally except cycloheximide (Lane B). See Table IV for further details. SDS-polyacrylamide gel (10%) electrophoresis of the microsomes was carried out as described in Materials and Methods. The dried slab gel was exposed to Kodak AR 'X-Omat' film at $-70\,^{\circ}$C. About 30 μg of protein were applied.

Fig. 9. Autoradiogram of [^{35}S]methionine-labeled gastric mucosal microsomes. The microsomes were prepared from the rats fasted overnight and treated with 100 μCi of [^{35}S]-methionine (Lane A), from those treated additionally with carbachol (Lane B), or the microsomes from those successively treated with cycloheximide, [^{35}S]methione and carbachol (Lane C) or with [^{35}S]methionine and carbachol (Lane D). The latter autoradiogram was of lighter intensity probably because of a combination of lesser proteins applied and a shorter period of exposure. Other experimental conditions were the same as described in the legend of Fig. 6.

resting or the carbachol-stimulated rats (Fig. 9). No other radioactive polypeptides were detected in autoradiograms of the gastric mucosal homogenates within an hour of [^{35}S]methionine injection. Cycloheximide also blocked incorporation of the radioactivity into the 14 kDa polypeptides (Fig. 9).

Discussion

Studies on the involvement of de novo protein synthesis in gastric acid secretion yielded conflicting results. Generally, studies in vitro with amphibian gastric mucosa reported no significant inhibitory effect of puromycin and cycloheximide on acid secretion [16–18]. Also we have found that cycloheximide did not affect histamine-induced acid accumulation in isolated rabbit gastric glands (our unpublished results). In contrast, cycloheximide has been shown to inhibit acid secretion in the rat in several previous reports [19,20] as well as in our current study. Furthermore, we have established that cycloheximide partially blocked carbachol- or histamine-induced translocation of $(H^+ + K^+)$-ATPase from the intracellular reserve to the apical membrane of gastric parietal cells and induction of a KCl pathway in the latter. These interferences of the activation steps of the acid pump should have been responsible at least in part for the observed inhibition of gastric acid secretion by cycloheximide in vivo (Fig. 1). Several observations made in this study indicate that these activities of cycloheximide are due to its inhibition of synthesis of a specific polypeptide(s) involved in the critical steps of gastric acid secretory process: (1) The drug was effective when given at least 30 min before carbachol, but showed no effect when given at the same time with or 30 min after carbachol. It should be noted that carbachol did not diminish the ability of cycloheximide to inhibit protein synthesis (see Table III). Therefore the disappearance of the inhibitory effect of cycloheximide as the drug was injected with or after carbachol could not be related to its absorption. Rather, it could be logically explained by the proposal that nascent polypeptides are required in the early stage of carbachol-mediated intracellular processes. (2) Puromycin, another inhibitor of protein synthesis but of a chemical structure different from cycloheximide, also disrupted the carbachol-initiated activation processes of the acid pump. (3) Actinomycin D and U-17701, which is a structural analog of cycloheximide without the ability to inhibit protein synthesis, had no effect. (4) Cycloheximide was active against carbachol as well as against histamine. Finally our earlier study [21] has shown that cycloheximide had no noticeable effect on $(H^+ + K^+)$-ATPase activity in rat gastric mucosa within 3 h. Further, our current observation that cycloheximide did not perturb either the distribution pattern of $(H^+ + K^+)$-ATPase or valinomycin- and K^+-dependent H^+ uptake in the resting state emphasizes the specificity of the cycloheximide action and its lack of broad toxicity. It appears, therefore, that the secretagogue-induced acid secretory process in vivo employs much more complex systems, including de novo protein synthesis of nascent polypeptides among others, than the similar process observed in vitro. Generally observed lower acid secretory rates in vitro as compared to those in vivo may well be due to the probable differences in their respective secretory mechanisms.

Our search for nascent polypeptides likely involved in the acid secretory process had led to the discovery of two most actively synthesized polypeptides in the rat gastric mucosa; the 38 and 14 kDa polypeptides. The larger polypeptide has been identified as rat pepsinogen. The identity of the smaller polypeptide is not known, but its several characteristics are noteworthy. This polypeptide appears to be a constitutive protein of a very short half-life, since it was actively synthesized both in the resting and in the stimulated animals. Although the polypeptide was apparently associated with particulate fractions, it was not copurified with $(H^+ + K^+)$-ATPase (our preliminary observation) and therefore is not likely a component of KCl pathway or other possibly unknown transporters in the apical membrane of the parietal cell. Rather, if the smaller polypeptide were involved in acid secretion, its role would be in intracellular processes prior to that involving the apical membranes. It is well known that carbachol, a cholinergic agent, propagates its message through activation of various protein kinases and intracellular enzymes rather than stimulation of new protein synthesis. What has been shown in this study

is the possibility that the small polypeptide of a very short half life plays a key role in intracellular propagation of carbachol message and that its ready depletion with cycloheximide or puromycin, because of its short half life, may impair the intracellular process.

In this study, our attention has been limited to the effects of cycloheximide on the molecular changes in the apical membranes of gastric parietal cells. In view of the vital importance of protein synthesis in all cellular actions and of the complexity of the stomach functions, however, it is possible that cycloheximide may have affected other cellular actions beyond the scope of our study. We hope that this report revives the interest of researchers in this field in the role of gastric nascent polypeptides in acid secretion.

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