응용약물학회지, The Journal of Applied Pharmacology 5, 228-232(1997)

Pharmacological properties of the reversible inhibitor of the gastric H^+/K^+ ATPase, AU-164

Hyo Jung KIM[1], Eul Kgun YUM[2], Jong Kwon CHOI[2] and Hyae Gyeong CHEON[1]*

Korea Research Institute of Chemical Technology, Pharmaceutical Screening Center[1], Pharmaceutical Division[2], Taejon, 305-606, Korea

(Received April 10, 1997; accepted July 22, 1997)

Abstract – AU-164 was synthesized as a reversible gastric H^+/K^+ ATPase inhibitor, and its effects were tested in various systems. AU-164 inhibited rabbit gastric H^+/K^+ ATPase with an IC_{50} of 9 μM. On the other hand, AU-164 was a weak inhibitor for dog kidney Na^+/K^+ ATPase, indicating the selectivity for gastric H^+/K^+ ATPase. The reversible property of the AU-164-induced inhibition of H^+/K^+ ATPase was confirmed by filtering the inhibition mixture through Sephadex G-25M column. *In vivo* basal acid secretion was also inhibited by AU-164 under the pylorus ligation of Sprague-Dawley rats. In addition, AU-164 protected dose dependently gastric lesion induced by ethanol in rats. The ED_{50} value of 62 mg/kg *p.o.* was estimated. These results suggest that AU-164 is a potent, selective and reversible gastric H^+/K^+ ATPase inhibitor, and that AU-164 has a potential use for the clinical therapeutics of peptic ulcer disease.

Keywords□ H^+/K^+ ATPase, reversible inhibitor, basal acid secretion, gastric lesion, Na^+/K^+ ATPase

Peptic ulcer appears to result from overproduction of gastric acid and/or decrease in gastric mucosal defensive factors (Say and Sun, 1953). Consequently, reduction of gastric acid production has been an approach for peptic ulcer therapy. Among many ways in reducing gastric acid secretion, inhibition of gastric H^+/K^+ ATPase is a recently developed target for the invention of new peptic ulcer drugs.

Gastric H^+/K^+ ATPase, located in the apical membrane of the parietal cells, pumps proton out into the gastric lumen via energy driven from ATP hydrolysis (Sach *et al.*, 1976). Accordingly, the enzyme is involved in the secretion of gastric acid into the stomach. The gene encoding gastric H^+/K^+ ATPase was cloned from rat (Shull, 1990; Shull and Lingrel, 1986) and human (Maeda *et al.*, 1990), and its amino acid sequence revealed high homology to Na^+/K^+ ATPase. Like the Na^+/K^+ ATPase, gastric H^+/K^+ ATPase was composed of two subunits (α and β), and α subunit contains the catalytic component of the enzyme. The protein has M.W. of 114 kDa (α subunit) (Shull and Lingrel, 1986) and 33 kDa (β subunit) (Shull, 1990).

Many benzimidazole derivatives including omeprazole have been synthesized as irreversible gastric H^+/K^+ ATPase inhibitors. They bind to sulfhydryl group(s) of the enzyme resulting in the covalent linkage between the compound and the enzyme (Lorentzon *et al.*, 1985). Due to the irreversibility of the enzyme inactivation, they exhibit powerful and long lasting inhibition of the gastric acid secretion. On the other hand, they may have several disadvantages for clinical usages such as bacterial overgrowth resulting from extremely long lasting anacidity (Wingate, 1990; Larner and Lendrum, 1992), and possible carcinogenicity due to hypergastrinemia (Carlsson *et al.*, 1986).

Recent efforts from many research groups have turned on the development of reversible gastric H^+/K^+ ATPase inhibitors, and some of them are currently under clinical trials (Pope and Parsons, 1993). In our laboratory, the acylquinoline derivatives have been synthesized, and screened for their pharmacological properties as reversible H^+/K^+ ATPase inhibitors. We report here that AU-164 is an effective and reversible H^+/K^+ ATPase inhibitor *in vitro*, and inhibits *in vivo* gastric acid secretion, and that AU-164 is capable of reducing ethanol-induced gastric lesion with an ED_{50} value of 62 mg/kg *p.o.*

MATERIALS AND METHODS

Materials

*To whom correspondence should be addressed.

Adenosine 5'-triphosphate disodium salt (Na_2ATP), Na^+/K^+ ATPase (dog kidney), nigericin, trizma base (Tris), ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), magnesium chloride ($MgCl_2$), ammonium chloride (NH_4Cl), bovine serum albumin (BSA), sodium chloride (NaCl), potassium chloride (KCl), dimethylsulfoxide (DMSO), sucrose, and (N-[2-hydroxyethyl]piperazine-N-[2-ethane-sulfonic acid]) (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bio Rad dye reagent concentrate was purchased from BioRad Laboratories (Richmond, CA, USA). PD-10 columns (1.5×5 cm) prepacked with Sephadex G-25M were purchased from Pharmacia Chemical Co. (Piscataway, NJ, USA). Amicon-30 concentrators were obtained from Amicon Corp. (Danvers, MA, USA). Polyethylene glycol 400 (PEG 400) and perchloric acid ($HClO_4$, 60%) were purchased from Junsei Chemical Co. (Japan). Butylacetate and carboxymethylcellulose (CMC) were purchased from Showa Chemical Co. (Japan). Diethylether was obtained from Oriental Chemical Industry (Korea). Formaldehyde (37%) was obtained from Merck Co. (Germany). AU-164 was synthesized by us and the synthetic procedures will be published elsewhere.

Enzyme preparation

H^+/K^+ ATPase was prepared from the fundic mucosae of New Zealand White Rabbits (2-3 kg, male) as described previously (Cheon *et al.*, 1995). The mucosal layer of the gastric fundus was scraped, and homogenized in 40 mM Tris/HCl, pH 7.4 containing 0.25 M sucrose, 2 mM HEPES, 2 mM $MgCl_2$, 2 mM EDTA. The homogenate was centrifuged at 10,000×g for 30 min at 4℃. The resulting supernatant was recentrifuged at 100,000×g for 60 min at 4℃. The pellets were resuspended in 40 mM Tris/HCl buffer (pH 7.4), and stored at -70℃. The protein concentration of the preparation was determined by the method of Bradford (Bradford, 1976).

H^+/K^+ ATPase assay

H^+/K^+ ATPase activity was determined as follows. Enzyme preparation (20 μg protein) was preincubated at 37℃ water bath for 30 min in 200 μl of a medium containing 40 mM Tris/HCl (pH 7.4) buffer, 4 mM $MgCl_2$, 5 μg/ml nigericin in methanol, and with or without AU-164 in DMSO. The assay reaction was initiated by adding 6.7 mM Na_2ATP (50 μl), continued for 30 min, and terminated by addition of 30% cold TCA (50 μl). The reaction mixture was centrifuged, and the released inorganic phosphate in the supernatant was measured spectrophotometrically (Yoda and Hokin, 1970). Specific H^+/K^+ ATPase activity was determined by the difference between the activities in the absence and in the presence of 48 mM KCl and 6 mM NH_4Cl.

Na^+/K^+ ATPase assay

The reaction mixture contained 2 mM $MgCl_2$, 2 mM Na_2ATP, 40 mM Tris/HCl, pH 7.4, and 20 μg Na^+/K^+ ATPase (Sigma), with or without 100 mM NaCl and 10 mM KCl (250 μl total volume). The reaction was started by adding AU-164 in DMSO (5 μl). After 30 min incubation at 37℃, the reaction was stopped by adding 30% cold TCA. The enzyme activity was assayed by measuring the released inorganic phosphate from ATP according to Yoda and Hokin (1970).

Reversibility of the AU-164-induced inactivation of the H^+/K^+ ATPase activities

Enzyme preparation (0.55 mg/ml) was incubated with either DMSO alone or AU-164 (0.5 mM final) in DMSO at 37℃ for 30 min. Final concentration of DMSO in the incubation mixture was 2%, which did not affect control enzyme activity. Aliquot (10 μl) was taken out at the end of the incubation and H^+/K^+ ATPase activity was assayed as described above. The remaining portion of the incubation mixture was passed into PD-10 desalting column, and the eluant containing the enzyme preparation was collected, and concentrated by using Amicon-30 concentrator. The protein concentration of the eluant was determined by the Bradford method (1976), and adjusted for H^+/K^+ ATPase assay. The enzyme activity was determined as described above.

Effect of AU-164 on the basal gastric acid secretion

In vivo antisecretory effect was examined according to Shay *et al.* (1954). Sprague-Dawley rats (150-250 g, male), obtained from Charles River (Japan), were fasted for 24 hr before experiment. The pyloric portion was ligated under diethylether anesthesia. AU-164 (20 mg/kg) in PEG 400 suspension or PEG 400 alone was administered intraduodenally. Five hr after the surgery, the stomach was isolated, and the accumulated gastric juice was collected. The gastric content was analyzed for gastric acid volume, pH and acid output by using Orion 960 autochemistry analyzer.

Effect of AU-164 on the ethanol (95%)-induced gastric lesion

SD rats were fasted for 24 hr before experiment. AU-164 in 0.5% carboxy methylcellulose (CMC) was orally administered, and 95% ethanol (1 ml/rat) was administered

p.o. 1 hr later. The stomach was isolated 1 hr after ethanol administration, and inflated with the injection of 1% formalin (13 ml). After fixing in 1% formalin for 1 hr, the greater curvature of the stomach was opened, and examined the gastric lesion macroscopically. The ulcer lesion (mm) was measured, calculated as the sum of length of the lesions, and compared with the control group treated with 0.5% CMC alone.

Statistical analysis

All statistical evaluations were done by Students *t*-test. The protection ratio on the ethanol-induced gastric lesion was calculated as follows:

$$\text{Protection ratio (\%)} = \frac{\text{ulcer lesion of control group} - \text{ulcer lesion of drug treated group}}{\text{ulcer lesion of control group}} \times 100$$

RESULTS

Effects of AU-164 on gastric H^+/K^+ ATPase *in vitro*

To develop novel and effective antiulcer drugs, various acylquinoline derivatives were synthesized and tested for their activity. As a primary screening system, effects on gastric H^+/K^+ ATPase *in vitro* were examined. As shown in Fig. 1, AU-164 inhibited gastric H^+/K^+ ATPase in a concentration dependent manner. Its IC_{50} value was estimated to be 9 μM.

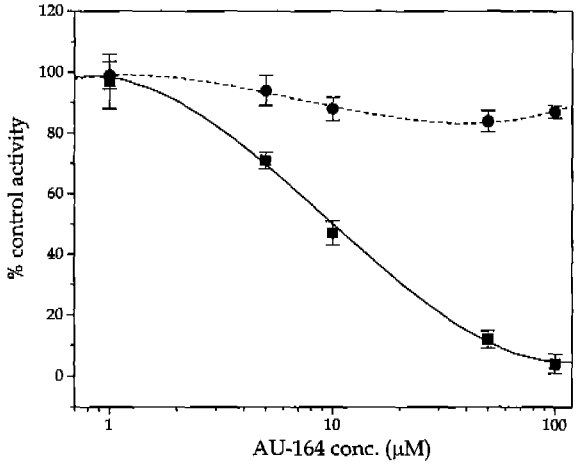

Fig. 1. Effects of AU-164 on H^+/K^+ ATPase and Na^+/K^+ ATPase activities. Either H^+/K^+ ATPase (■) or Na^+/K^+ ATPase (●) was treated with various concentrations of AU-164, and the remaining activities were determined as described in Materials and Methods. Results are expressed as percent control activity which was incubated with DMSO alone. Each data represents mean±SD of three separate experiments.

In order to examine the selectivity of AU-164-mediated inhibition of H^+/K^+ ATPase, the effects of AU-164 on Na^+/K^+ ATPase, a related ATPase were examined. The inhibitory activity of AU-164 on Na^+/K^+ ATPase was very weak as compared with that on gastric H^+/K^+ ATPase (Fig. 1, ED_{50}>100 μM), despite the similarity of the amino acid sequence between two ATPases. These results suggest that AU-164 is a selective and effective inhibitor of gastric H^+/K^+ ATPase.

Since AU-164 was postulated to be a reversible inhibitor, the reversibility of AU-164-mediated inhibition of H^+/K^+ ATPase was investigated. Reaction mixture was filtered through Sephadex G-25M column and the H^+/K^+ ATPase activity was determined. Gastric H^+/K^+ ATPase activity was recovered up to 90% upon filtration, confirming the reversible nature of the inactivation (Fig. 2). As a negative control, omeprazole-treated enzyme mixture (known as irreversible nature of the inactivation) was passed into Sephadex G-25M column, and the inactivation of the enzyme activity was not recovered.

Effects of AU-164 on *in vivo* acid secretion and on the ethanol-induced gastric lesion

Sprague-Dawley rats were ligated in pylorus region under diethylether anesthesia. Intraduodenal administration of AU-164 (20 mg/kg) resulted in the reduction of basal acid secretion (Fig. 3). Total acid output, acid concentration and gastric juice volume all were decreased significantly as compared with control treatment. SK&F 96067,

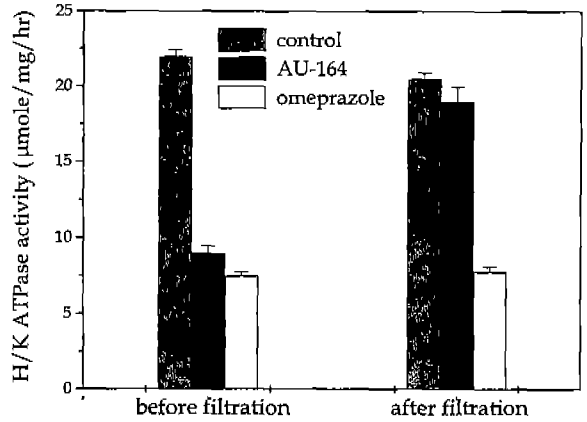

Fig. 2. Reversibility of AU-164-induced inactivation of H^+/K^+ ATPase activity. H^+/K^+ ATPase was incubated with either AU-164 (0.5 mM) or omeprazole (0.5 mM). Enzyme activity was determined before and after filtration of incubation mixture through Sephadex G-25M column. Results represent mean± SD of three separate experiments.

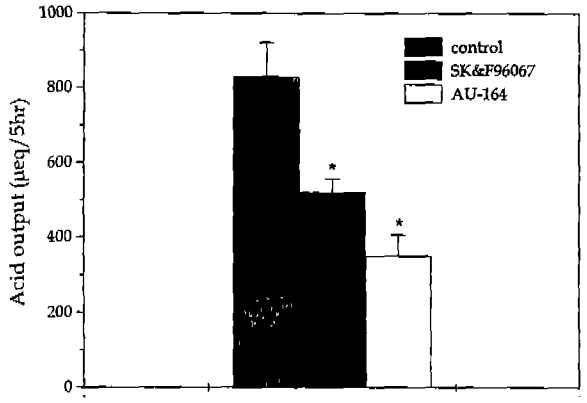

Fig. 3. Effect of AU-164 on the basal gastric acid secretion. Either AU-164 (20 mg/kg) or SK&F 96067 (20 mg/kg) was administered intraduodenally into SD rats with pylorus ligation. Gastric content was analyzed 5 hr after the surgery, and acid output was determined from the volume and acidity of gastric juice. Results represent mean±SD of three separate experiments. *P<0.05 vs control

a known reversible inhibitor also inhibited basal acid secretion to 50% of the control. By comparison, omeprazole administration with the same dose produced 60 to 70% inhibition of the basal acid secretion.

As shown in Fig. 4A, ethanol (95%) administration produced severe band-like mucosal lesions in the grandular stomach. AU-164 treatment reduced mucosal lesions, complete prevention being achieved at 300 mg/kg *p.o.* (Fig. 4B). The lesion index in the control group (ethanol administration alone) was 65.5 mm. AU-164 protected gastric mucosal damage dose dependently with significant protection at 100 mg/kg *p.o.* The ED_{50} value was 62 mg/kg *p.o.* (Table I).

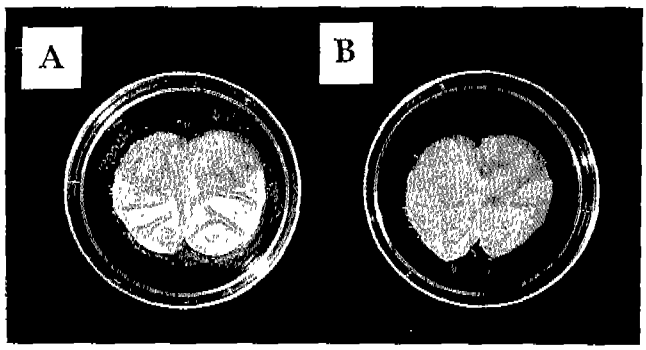

Fig. 4. Effect of AU-164 on the ethanol-induced gastric lesion. AU-164 (300 mg/kg) was orally administered into SD rats, and 95% ethanol (1 ml) was administered *p.o.* 1 hr later. One hour after the ethanol administration, the stomach was isolated, and gastric lesion was measured macroscopically. A. Gastric lesion induced by 95% ethanol. B. Treatment of the ethanol-induced gastric lesion by AU-164.

Table I. Effect of AU-164 on the ethanol-induced gastric lesion in rats

Dose (mg/kg)	Ulcer lesion[a] (mm)	Protection ratio[b] (%)
0	65.5±33.1	
30	53.2±52.1	18.8
100	16.4±18.0*	75.0
300	5.00±3.46*	82.4

[a]AU-164 was administered orally followed by oral administration of 95% ethanol (1 ml/rat). The rats were sacrificed 1 hr later and the stomach was removed. The length of ulcer lesion was measured macroscopically after fixation in formalin. Ulcer lesion was expressed as mean±SD (N=7). [b]Protection ratio was calulated as described in Materials and Methods. *P<0.05 vs control (0 mg/kg).

DISCUSSION

Reversible gastric H^+/K^+ ATPase inhibitors have recently been considered as important therapeutic candidates for peptic ulcer diseases. They act on the gastric H^+/K^+ transporting ATPase which is involved in the final step in the secretion of gastric acid. The reversibility of the enzyme inhibition may confer several clinical benefits over covalent H^+/K^+ ATPase inhibitors such as omeprazole. These include better control of dosing, less problems of bacterial overgrowth (Wingate, 1990), hypergastrinemia and carcinoid lesions in stomach (Carlsson *et al.*, 1986). In fact, some of the reversible H^+/K^+ ATPase inhibitors are currently in clinical development for therapeutic use (Pope and Parsons, 1993).

In making efforts to develop reversible gastric H^+/K^+ ATPase inhibitors for novel antiulcer agents, the acylquinoline derivatives were synthesized and examined for their activity. AU-164 was a promising compound being superior in potency among them. *In vitro* system, AU-164 exhibited expected reversible inactivation of H^+/K^+ ATPase. It has a potent inhibitory activity on gastric H^+/K^+ ATPase (IC_{50}=9 μM). By comparison, IC_{50} of SK&F 96067, a prototype of reversible inhibitors was 20 μM in our experimental conditions. Furthermore, the effect of AU-164 on Na^+/K^+ ATPase, a closely related ATPase was weak (IC_{50}>100 μM) indicating a high selectivity for the H^+/K^+ ATPase. Accordingly, the high intrinsic selectivity of AU-164 for H^+/K^+ ATPase may add benefit as a therapeutic agent.

Using *in vivo* experimental animal systems, AU-164 has been shown to be an effective inhibitor on basal acid secretion when administered intraduodenally. The potency

of AU-164 at 20 mg/kg was 1.5 fold greater than that of SK&F 96067. AU-164 inhibited gastric acid secretion more than 50% at 20 mg/kg in Shay model. By contrast, ED_{50} of AU-164 on the ethanol-induced gastric damage was 62 mg/kg. This is comparable with that of SK&F 96067 (ED_{50}=75 mg/kg) used as a reference drug. The inhibition by AU-164 of gastric lesions appears to be complete at 300 mg/kg *p.o.*

Ulcerogenesis induced by ethanol is related to the impairment of mucous and mucosal circulation (Kuwata *et al.*, 1985; Trier *et al.*, 1987; Oates and Hakkinen, 1988). Indeed, it has been observed that mucous formation was reduced by ethanol treatment (unpublished observation). Though AU-164 mainly acts as an inhibitor of gastric acid secretion, the detailed mode of the protective effect of AU-164 against ethanol is currently under investigation.

It has been reported that one of the drawback of the reversible inhibitors is the toxicity against stomach and liver (Long *et al.*, 1983; Wallmark *et al.*, 1987). To investigate toxic effect of AU-164, the compound (300 mg/kg) was orally administered into rats for three weeks. The subchronic treatment of AU-164 showed no apparent change in terms of organ weight and histological examination (results not shown).

In conclusion, the present study demonstrates that AU-164 possesses pronounced antiulcer activity. Detailed studies on toxicology as well as pharmacokinetics of AU-164 and the effects of the compound on the defensive factors such as prostaglandin and mucous formation are in progress.

REFERENCES

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Carlsson, E., Larsson, H., Mattsson, H., Ryberg, B. and Sundell, G. (1986). Pharmacology and toxicology of omeprazole-With special reference to the effects on the gastric mucosa. *Scand. J. Gastroenterol.* **21** (118) Suppl., 31-38.

Cheon, H. G., Kim, H. J., Yum, E. K., Cho, S. Y., Kim, D. Y. and Yang, S. I. (1995). Studies on the mechanism of action of the gastric H^++K^+ ATPase inhibitor KH 3218. *J. Applied Pharmacol.* **3**, 205-209.

Kuwata, H., Ishihara, K., Kakei, M., Ohara, S., Okabe, H. and Hoffa, K. (1985). Correlation of quantitative changes of gastric mucosal glycoproteins with ethanol-induced gastric damage in rats. *Japan J. Gastroenterol.* **82**, 28-33.

Larner, A. J. and Lendrum, R. (1992). Oesophageal candidiasis after omeprazole therapy. *Gut* **33**, 860-861.

Long, J. F., Chiu, P.J.S., Derelanko, M. J. and Steinberg, M. (1983). Gastric antisecretory and cytoprotective activities of SCH 28080. *J. Pharmacol. Exp. Ther.* **226**, 114-120.

Lorentzon, P., Eklundh, B., Brandstrom, A. and Wallmark, B. (1985). The mechanism for inhibition of gastric H^++K^+ ATPase by omeprazole. *Biochim. Biophys. Acta* **817**, 25-32.

Maeda, M., Oshiman, K. I., Tamura, S. and Futai, M. (1990). Human gastric (H^++K^+)-ATPase gene. Similarity to (Na^++K^+)-ATPase genes in exon/intron organization but difference in control region. *J. Biol. Chem.* **265**, 9027-9032.

Oates, P. J. and Hakkinen, J. P. (1988). Studies on the mechanisms of ethanol-induced gastric damage in rats. *Gastroenterology* **94**, 10-21.

Pope, A. J. and Parsons, M. E. (1993). Reversible inhibitors of the gastric H^+/K^+ transporting ATPase: A new class of antisecretory agent. *Trends Pharmacol. Sci.* **14**, 323-325.

Sachs, G., Chang, H. H., Rabon, E., Schackman, R., Lewin, M. and Saccomani, G. (1976). A nonelectrogenic H^+ pump in plasma membranes of hog stomach. *J. Biol. Chem.* **251**, 7690-7698.

Say, H. and Sun, D.C.H. (1953). In *Gastroenterology* (E. H. Rockus, Ed.), Saunders Co., Philadelphia-London.

Shay, M., Sun, D.C.H. and Gruenstein, M. (1954). A quantitative method for measuring spontaneous gastric secretion in the rat. *Gastroenterology* **26**, 906-913.

Shull, G. E. (1990). cDNA cloning of the β-subunit of the rat gastric H,K-ATPase. *J. Biol. Chem.* **265**, 12123-12126.

Shull, G. E. and Lingrel, J. B. (1986). Molecular cloning of the rat stomach (H^++K^+)-ATPase. *J. Biol. Chem.* **261**, 16788-16791.

Trier, J. S., Szabo, S. and Allan, C.H. (1987). Ethanol-induced damage to mucosal capillaries of rat stomach. Ultrastructural features and effects of prostaglandin F2 and cysteamine. *Gastroenterology* **92**, 13-22.

Wallmark, B., Briving, C., Fryklund, J., Munson, K., Jackson, R., Mendlein, J., Rabon, E., and Sachs, G. (1987). Inhibition of gastric H^++K^+-ATPase amd acid secretion by SCH 28080, a substituted pyridyl(1,2a)imidazole. *J. Biol. Chem.* **262**, 2077-2084.

Wingate, D. L. (1990). Acid reduction and recurrent enteritis. *Lancet* **335**, 222.

Yoda, A. and Hokin, L.E. (1970). On the reversibility of binding of cardiotonic steroids to a partially purified (Na+K)-activated adenosinetriphosphatase from beef brain. *Biochim. Biophys. Res. Commun.* **40**, 880-886.