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ETHANOL, ITS EFFECT ON THE SYNTHESIS OF PROTEINS BY GUINEA-PIG GASTRIC MUCOSA

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Abstract—Incorporation of L-[U-^{14}C] leucine into proteins is taken to indicate the synthesis of proteins by guinea pig gastric mucosa.

Ethanol reduced the synthesis of proteins *in vitro* by homogenized mucosa, by isolated gastric epithelial cell preparations and by intact tissue.

Intact stomach wall incubated without ethanol in phosphate buffered saline showed progressively increasing incorporation of the precursor into tissue proteins and into proteins which were secreted into the mucosal incubation media. On isopycnic CsCl gradient fractionation radioactive tissue proteins were found at the top of the gradient (fraction L1, sp.gr.1.11–1.20) while radioactive secreted proteins sedimented to the bottom of the gradient as the carbohydrate rich high density gastric mucosal glycoprotein fraction L3 (sp.gr.1.29–1.33).

Ethanol significantly but reversibly reduced the incorporation of radioactive leucine by intact mucosa into both the tissue proteins and the secreted proteins. Uptake of the precursor into the intracellular acid soluble pool was not impaired by ethanol and no significant differences were detected in the specific activities of free intracellular leucine between the ethanol treated samples and the corresponding controls.

It is suggested that the ulcerogenic nature of ethanol may be associated with inhibition of the synthesis of proteins within mucosal epithelium leading to reduction in the output of mucosal secretory glycoproteins with subsequent impairment of the cytoprotective properties of the dynamic mucous barrier.

Although adverse effects of alcohol are extensively studied local effect of alcohol on gastrointestinal tissue metabolism remains relatively unexplored (see [1, 2] for reviews).

High concentrations of alcohol (20%–40%) administered into the stomachs of dogs, rats and mice were shown to cause multiple gastric erosions, to decrease superficial mucus layer and mucin content of surface epithelial cells [3, 4] and to cause cell damage as assessed by ultrastructural studies [5]. Instillation of absolute alcohol into rat stomachs produced areas of gross hemorrhagic necrosis of the mucosa [6].

In *in vitro* experiments reduction of galactosamine synthesis by human gastric mucosa [7] and inhibition of the incorporation of radioactive glucosamine into gastric mucosal glycoproteins [8] by alcohol have also been reported.

The aim of our work was to determine the effect of low concentrations of ethanol on the synthesis of proteins by gastric mucosal epithelium, especially on the synthesis of the protein core of the glycoproteins of gastric mucus. Impairment of the synthesis of mucosal glycoproteins before their glycosylation and secretion would be expected to lead to a defective mucous barrier. Local effects of mucosal irritants such as of ethanol, and as previously demonstrated of salicylates [9], on epithelial cell metabolism may well account for their ulcerogenicity.

MATERIALS AND METHODS

Reagents

Samples were incubated either in unsupplemented Dulbecco "A" phosphate buffered saline (PBS, pH 7.3–7.5, Oxoid Ltd., U.K.: 2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4) or in Dulbecco's modified Minimum Essential Medium (MEM: pH 7.2 GIBCO Europe Ltd., containing glucose (1000 mg/l, sodium pyruvate (110 mg/l), a range of amino acids, including 802 nmol/ml leucine, and of vitamins.

L-[U-^{14}C] leucine and [G-^{3}H] Dansyl chloride were from Radiochemical Centre, Amersham, U.K.

All other chemicals were from BDH Chemicals Ltd. (Poole, U.K.)

Experimental procedures

Adult male guinea pigs between 700 g and 1000 g in weight were killed by decapitation and antral parts of the stomachs used.

Incubation of tissue homogenates. Gastric epithelium stripped of the sub-mucosal layers was homogenized in phosphate buffered saline and 1 ml aliquots incubated while shaking for 1 hr at 37° with radioactive leucine.

Gastric epithelial cell preparations. Epithelial cell sheets released from the sub-mucosal layers of the stomach wall by incubation for 15 min at 37° in 10 mM EDTA in phosphate buffered saline [9, 10] were disrupted into smaller fragments by passing through nylon stocking mesh, sedimented, washed, resuspended as required and dispensed.

One-millilitre samples in phosphate buffered saline were incubated while shaking for 1 hr with L-[U-^{14}C] leucine at 37°.

The suspensions contained fragments of mucosal epithelium. Specific cell types were not characterized

and it was assumed that most cells of normal epithelium were represented. Earlier work [9] has shown such cell suspensions to be viable in terms of protein synthesis for up to 4 hours of *in vitro* incubation.

Intact tissue incubations. Pieces of intact stomach wall (113 mm^2, containing between 10 mg and 12 mg protein per sample) were mounted in Ussing-type leucite chambers and incubated at 37° in media circulated independently on the luminal and the external side and gassed with O_2/CO_2 (3%). Four tissue samples/animal (1 control and 3 ethanol samples) were incubated simultaneously in each experiment. L-[U-^{14}C] leucine and ethanol were routinely added to the *mucosal media* which bathed the luminal side of the mucosa.

In order to test the reversibility of the effect of ethanol on protein synthesis tissue samples were preincubated for periods of 1 and of 4 hr with 5% ethanol. The ethanol containing media were replaced with phosphate buffered saline and the samples incubated for a further period of 4 hr with radioactive leucine.

After incubation, tissue samples were washed, the mucosae scraped, scrapings homogenized and proteins precipitated with trichloroacetic acid.

^{14}C activity of the supernatants represents total intracellular acid-soluble pool activity; the activity of intracellular leucine was estimated after thin layer chromatography and its specific activity determined after dansylation of amino acids of the total acid soluble pool with [G-^{3}H] dansyl chloride.

Secreted proteins were isolated from experimental mucosal media by precipitation with trichloroacetic acid. All media bathing the external side of the tissue were discarded; these solutions contained no measurable ^{14}C activity at the end of incubation.

Isopycnic CsCl *gradient fractionation*. Proteins suspended in Tris–HCl buffer (0.1 M, 5 mM EDTA, pH 8.3) were mixed with solid CsCl (0.425 g/ml suspension), centrifuged at 4° for 72 hr at 100,000 *g* and 1 ml layers collected. This procedure separates high molecular weight glycoproteins which sediment at the bottom of the gradient (fraction L3, sp.gr. 1.29–1.33) from the remaining proteins of the mucosa [11, 12].

Analytical methods

Specific activity of proteins. Proteins isolated from the suspensions by precipitation with 10% trichloracetic acid and centrifugation were estimated on the Technicon Autoanalyser by the method of Lowry *et al.* [13] after 18 hr hydrolysis at 37° in 0.5 N NaOH. Aliquots of the hydrolysed solutions were also counted.

Carbohydrates. Hexose content of aqueous samples was determined by the method of Dubois [14] using glucose as standard. Carbohydrate levels indicate only the relative amounts hexose found in different samples. They cannot be treated as absolute since the sugars which are associated with gastric mucosal glycoproteins comprise mixtures of a number of different carbohydrates [12] all of which contribute in different yields to the intensity of colour produced by the colorimetric reaction used.

Radioactivity was determined by counting in Liquid Scintillator Unisolve 100 (Koch-Light Laboratories Ltd., England) in a Beckman model LS 100 or an LKB Liquid Scintillation counter set to 1% error. Counts below twice the background were rejected.

The efficiency of counting of ^{14}C activity in single isotope samples was either 94% in the Beckman counter or 76% in the LKB counter. The efficiency of counting of dual isotope dansyl leucine was 25% for ^{14}C with 42% overspill into the 3H channel, and 10% for 3H.

Thin-layer chromatography. Acid soluble fractions of intact tissue homogenates extracted with ether and lyophilized were quantitatively applied to Merck pre-coated Cellulose plates together with non-radioactive leucine marker. Chromatograms were developed in (i) *n*-butanol/acetic acid/water (12:3:5 v/v/v), (ii) ethanol/ammonia/water (20:1:4 v/v/v), and (iii) *n*-butanol/pyridine/water (1:1:1 v/v/v), and sprayed with 0.5% ninhydrin in *n*-butanol saturated with water. Spots were scraped as soon as they became visible (5–10 min), the powder suspended in 1 ml 0.1 N HCl, mixed with 5 ml Unisolve 100 and counted. The efficiency of counting of the suspensions was 76% and recovery of total activity from the plates 91.8 ± 9.2% (57).

Specific activity of intracellular leucine was determined by a modified method of Airhart *et al.* [15, 16].

Lyophilized acid soluble residues in 0.1 ml 1 M $NaHCO_3$ were mixed with 0.1 ml [G-^{3}H] dansyl chloride in acetone [2 mM solution, spec.act. 20 mCi (740 MBq)/mmol], incubated at 37° for 90 min, mixed with 2 ml acetone and centrifuged. The supernatants were evaporated to dryness with a stream of nitrogen and residual dansylated amino acids separated by two dimensional chromatography on polyamide layer sheets (BDH Chemical Ltd.) in (i) formic acid : water (3 : 100 v/v), and (ii) benzene : glacial acetic acid (90 : 10 v/v).

Dansyl leucine spots viewed under short wave u.v. and identified by co-chromatography with non-radioactive marker were cut out, suspended in 0.1 N HCl, mixed with Unisolve 100 and counted.

Statistical significance of the differences in values expressed as probability P was calculated by Student's *t*-test. Results are presented as the mean ± standard deviation of the number of experimental samples given in parentheses and include experimental error and biological variations.

RESULTS AND DISCUSSION

Ethanol reduced *in vitro* incorporation of radioactive leucine into proteins of guinea pig gastric mucosa by (i) gastric mucosal tissue homogenates (Table 1), (ii) isolated epithelial cell preparations (Fig. 1), and (iii) intact gastric mucosa. In all three systems the degree of inhibition increased with increasing concentration of ethanol.

Intact tissue experiments

Four hours was chosen as the standard period of incubation of intact mucosa with radioactive leucine in all routine experiments.

Uptake of L-[U-^{14}C] leucine into the intracellular acid soluble pool reached a constant level after an

Table 1. Incubation of homogenized mucosa. ^{14}C activity of proteins

	pmol Leucine incorporated/ mg sample protein	P
Control samples	0.317 ± 0.076 (20)	Control
5% Ethanol	0.138 ± 0.060 (20)	<0.001
10% Ethanol	0.103 ± 0.025 (20)	<0.001

Incorporation of L-[U-^{14}C] leucine [spec. act. 330 mCi (12.21 GBq)/mmol, 303 pmol/ml] into proteins by 1 ml homogenate samples [1885 ± 530 μg protein/ml (60)] incubated for 1 hr in phosphate buffered saline.

initial period of equilibration (around 1 hr) and remained at that level for the duration of the experiment (Fig. 2A). After 4 hr the level of activity of the soluble pool, elevated by ethanol (Table 2), was related to the concentration of the precursor in the incubation medium in both the control and the ethanol treated samples (Fig. 2B).

No significant differences were detected in the specific activities of free intracellular leucine between the ethanol treated and the corresponding control samples either at dilute concentrations of the precursor (202 pmol/ml) in phosphate buffered saline, or at higher concentrations (802 nmol/ml) in Dulbecco's minimum essential medium (Table 2).

Specific activities of intracellular leucine were not determined in recovery experiments; when calculating incorporation of the precursor in nmol the specific activity of routine controls was used.

In contrast to the uptake of radioactive leucine from the medium into the acid soluble pool ethanol significantly reduced the incorporation of the precursor into tissue proteins and into proteins which were secreted into the mucosal media.

The specific activities of proteins of control samples increased throughout the whole period of incubation, in some experiments up to 8 hr (unpublished observation), indicating active incorporation of the precursor, i.e. viability of the tissue in terms of protein synthesis (Fig. 3). Ethanol significantly suppressed the incorporation of the precursor into both the tissue proteins and the secreted (mucosal media) proteins (Tables 3–5).

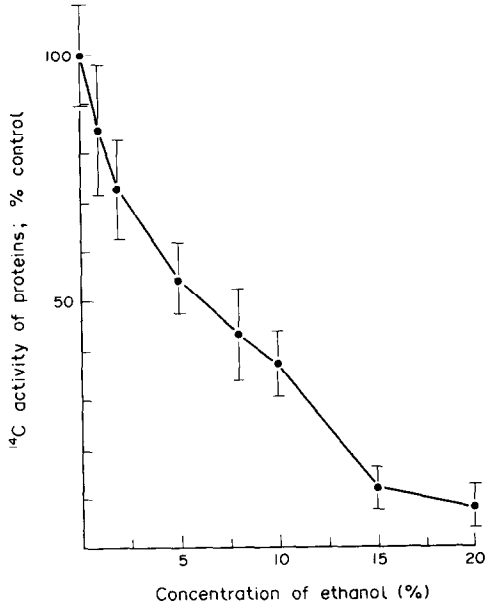

Fig. 1. Isolated gastric epithelial cell preparations. The effect of ethanol on protein synthesis. One-millilitre cell samples (1.0–1.5 mg protein) were incubated for 1 hr with L-[U-^{14}C] leucine [spec. act. 330 mCi (12.21 GBq)/mmol, 303 pmol/ml]. Results compiled from 7 separate experiments (5 samples per point per experiment) are given as % activity of the corresponding controls.

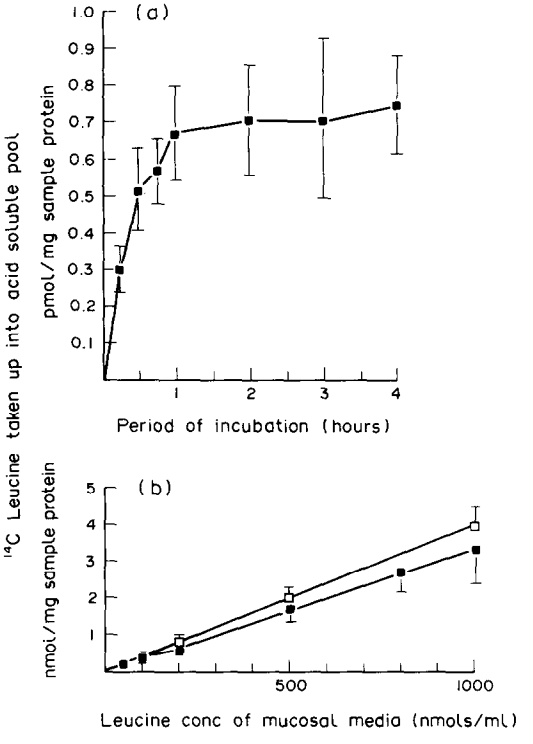

Fig. 2. Intact tissue incubations. Acid soluble pool. Uptake of radioactive leucine from the media by intact tissue samples incubated in phosphate buffered saline. Molecules of leucine taken up from the media into the total soluble intracellular pool per mg tissue protein: (A) as a function of time at a constant concentration of leucine (202 pmol/ml incubation medium); (B) as a function of concentration of leucine in the incubation media after 4 hours of incubation in control phosphate buffered saline (■—■) and in 5% ethanol (□—□).

Table 2. Intact tissue incubations. Intracellular leucine

	Activity/mg protein (d.p.m.)	Activity/mg protein (nmol)	P	Specific activity (d.p.m./nmol leucine)
PBS incubations				
Control	163 ± 53 (19)	0.22×10^{-3}	control	70.5 ± 18.3 (3)
5% Ethanol	244 ± 57 (7)	0.33×10^{-3}	<0.001	75.6 ± 11.4 (3)
10% Ethanol	482 ± 103 (4)	0.66×10^{-3}	<0.001	62.5 ± 23.6 (3)
MEM incubations				
Control	245 ± 35 (4)	1.324	control	35.4 ± 5.8 (3)
5% Ethanol	420 ± 77 (4)	2.270	<0.01	33.8 ± 2.9 (3)
10% Ethanol	544 ± 100 (4)	2.941	<0.01	31.1 ± 15.5 (3)

^{14}C activity of free intracellular leucine expressed as (i) d.p.m./mg sample protein and the equivalent molecules of leucine taken up from the mucosal media, and (ii) as the specific activity of free intracellular leucine in the samples.

4 hr incubations.

Concentration of leucine in the incubation media:

PBS incubations: 202 pmol/ml, spec. act. 330 mCi (12.21 GBq)/mmol;

MEM incubations: 802 nmol/ml, spec. act. 0.083 mCi (3.08 MBq)/mmol.

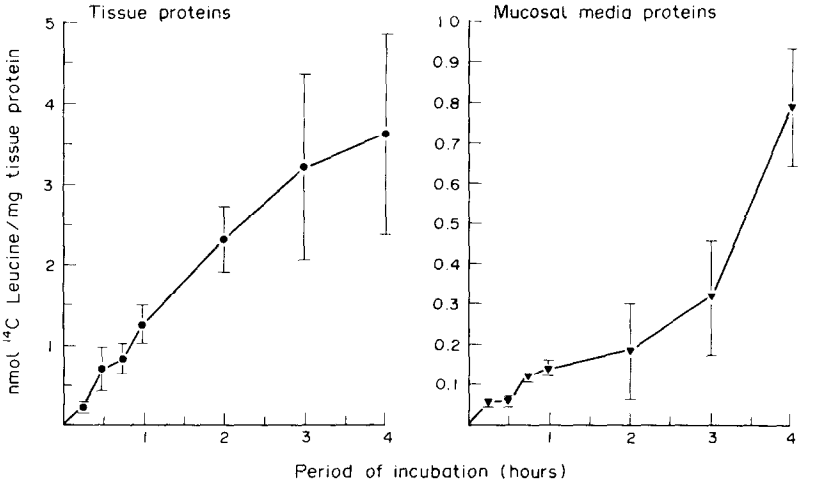

Fig. 3. Intact tissue incubations. Activity of proteins. Molecules of radioactive leucine of the mucosal media incorporated into tissue proteins and into secreted (mucosal media) proteins as a function of time. Incubations in phosphate buffered saline with L-[U-^{14}C] leucine [spec. act. 330 mCi (12.21 GBq)/ml, 202 pmol/ml].

Table 3. Intact tissue incubations. Tissue proteins

	Specific activity (d.p.m./mg)	P	Leucine (nmol/mg)
Control	3153 ± 1100 (26)	control	44.7 ± 15.6
5% Ethanol	1575 ± 605 (11)	<0.001	20.8 ± 8.0
10% Ethanol	107 ± 71 (4)	<0.001	1.7 ± 1.1
Recovery experiments			
1 hr pre-incubation:			
in control PBS	3334 ± 712 (7)	control	47.3 ± 10.1
in 5% Ethanol	2799 ± 1027 (13)	<0.1 (NS)	39.7 ± 14.6
4 hr pre-incubation:			
in control PBS	3950 ± 1487 (4)	control	56.0 ± 21.1
in 5% Ethanol	4038 ± 755 (4)	<0.4 (NS)	57.3 ± 10.7

4 hour incubation in phosphate buffered saline with L-[U-^{14}C]leucine [spec. act. 330 mCi (12.21 GBq)/mmol, 202 pmol/ml].

^{14}C activity of proteins expressed as specific activity (d.p.m./mg) and as molecules of leucine incorporated/mg protein, calculated from specific activities of *intracellular* leucine given in Table 2.

NS = not significant.

Table 4. Intact tissue incubations. Tissue proteins

	Specific activity (d.p.m./mg)	P	Leucine (nmol/mg)
Control	461 ± 164 (4)	control	13.0 ± 4.6
5% Ethanol	228 ± 89 (4)	<0.05	6.7 ± 2.6
10% Ethanol	36 ± 15 (4)	<0.001	1.2 ± 0.5

4 hr incubation in Minimum Essential Medium with L-[U-^{14}C] leucine [spec. act. 0.083 mCi (3.08 MBq)/mmol, 802 nmol/ml].

^{14}C activity of proteins expressed as specific activity (d.p.m./mg) and as molecules of leucine incorporated/mg protein calculated from specific activities of *intracellular* leucine given in Table 2.

Reduction in the specific activity of secreted proteins was associated with a reduction in hexose content of the material (Table 5).

Inhibition of the synthesis of proteins by ethanol was reversible even after 4 hr of pre-incubation of intact mucosa with 5% ethanol in unsupplemented phosphate buffered saline (Tables 3 and 5).

Isopycnic CsCl *gradient fractionation of proteins* (Fig. 4)

In both the control and the ethanol treated specimens radioactivity of *tissue proteins* was associated with the bulk of the material which sedimented at the top of the gradient (fraction L1, sp.gr. 1.11–1.20). These fractions contained over 96% of tissue proteins and of their ^{14}C activity in both the control and the ethanol treated samples but the specific activities of proteins of the latter were reduced.

The highly radioactive proteins of the mucosal media of control samples sedimented at the bottom of CsCl gradient (fraction L3, sp.gr. 1.29–1.33) together with most (over 79%) of the carbohydrates of the secreted material. This fraction is known to contain high density gastric mucosal glycoproteins [11, 12]. Ethanol reduced ^{14}C activity and hexose content of the material of this fraction to undetectable levels implying reduction in secretion of gastric mucosal glycoproteins.

GENERAL CONCLUSIONS

Ethanol reduced incorporation of radioactive leu-

Table 5. Intact tissue incubations. Secreted (mucosal media) proteins

	(d.p.m./mg)	^{14}C activity (nmol leucine/mg)	P	Protein (μg/medium)	Hexose (μg/medium)
Control	15472 ± 960 (16)	219.5 ± 13.6	Control	470 ± 155 (20)	294 ± 166 (10)
5% Ethanol	807 ± 440 (9)	10.7 ± 5.8	0.001	621 ± 193 (20)	19 ± 8 (8)
10% Ethanol	268 ± 205 (4)	4.3 ± 3.3	0.001	1229 ± 509 (9)	(0)
Recovery experiments					
1 hr pre-incubation:					
Control	12323 ± 8031 (7)	174.8 ± 113.9	Control	—	—
5% Ethanol	14959 ± 5854 (10)	212.2 ± 83.0	0.4 (NS)	—	—

4 hr incubation in phosphate buffered saline with L-[U-^{14}C]leucine [Spec. act. 330 mCi (12.21 GBq)/mmol, 202 pmol/ml].

^{14}C activity of proteins expressed as specific activity (d.p.m./mg) and as molecules of leucine incorporated/mg protein, calculated from the specific activities of *intracellular* leucine given in Table 2.

Protein and hexose content of the mucosal media are also given.

NS = not significant.

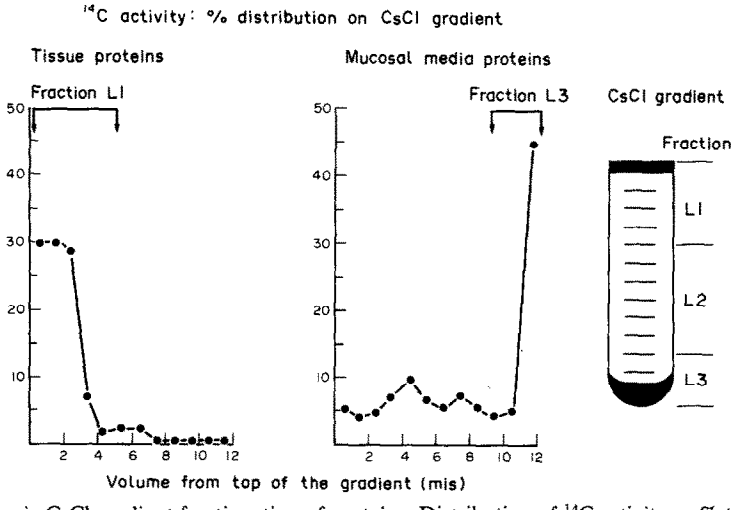

Fig. 4. Isopycnic CsCl gradient fractionation of proteins. Distribution of ^{14}C activity as % total activity of the sample: (A) tissue proteins, (B) mucosal media (secreted) proteins.

cine into proteins of guinea pig gastric mucosa during *in vitro* incubation while uptake of the precursor into the intracellular acid soluble pool was not impaired.

The synthesis of all proteins was reduced but the effect was most pronounced for the high density glycoprotein fraction of secreted proteins.

The mechanism responsible for the observed effects has not been elucidated although reduced availability of the precursor has been ruled out: no evidence emerged at any stage of our investigations of impairment of the uptake of radioactive leucine from the medium into the soluble pool of intact mucosa; moreover protein synthesis by mucosal homogenates, where the question of uptake of the precursor does not arise, was also reduced by ethanol.

The factors responsible for the control and regulation of the synthesis of proteins by gastric mucosa remain obscure and discussion of a possible mechanism involved in the effect of ethanol would be highly speculative. Also the relevance of our *in vitro* studies on guinea pig mucosa to the prevailing conditions in man remain to be established. It is of interest however to note that ethanol, which penetrates the mucosal barrier to reach the epithelium and be absorbed from the human stomach, may interfere with the metabolic process within the epithelial cells and in this way initiate degeneration of the established dynamic mucosal barrier against toxicity of the acidic contents of the lumen.

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