Choleragen (0.4 mg/ml) was incubated in 0.1 M $NaBO_3$ buffer pH 7.5 containing 5 mmoles of $CaCl_2$/l, and pronase at an enzyme/toxin ratio of 1/200, at 37 °C for 180 min.

Results and discussion. Pronase is able to digest fully the subunit A of choleragen, leaving the subunit B apparently unaffected. In fact, as shown in figure 1, the electrophoretic band corresponding to subunit A is no longer present in the sample deriving from the incubation mixture, while the subunit B shows the same pattern as that found in the sample of untreated toxin.

The ability to bind specific antibodies also remains unaltered by the treatment with pronase. As shown in figure 2, a reaction of complete identity is obtained in agar gel immunodiffusion between native and treated toxin. On the other hand, the toxicity of choleragen is reduced to only 15,000 blueing doses/mg after incubation with pronase.

These findings are consistent with the observation that the subunit A is a poor antigen[9], since the ability to bind antibodes raised against the whole toxin is not lost, although the subunit A is destroyed in our experiments. It has also been reported that the subunit A is essential for the toxicity, as it consitutes the effector moiety of choleragen[10]. This is in agreement with the reduced toxicity found after pronase incubation. The resistance of subunit B is not, generally speaking, a surprising finding. In fact many proteins have a core or a specific subunit that, in its native conformation, is not split by proteases. In our experiment this effect seems particularly pronounced.

Consequently, the limited proteolysis of choleragen with pronase could be of practical value for the preparation of the choleragen B subunit, to be used as an agonist to prevent the binding of choleragen to its receptors, or the provoke antitoxic immunity by including it in a cholera vaccine.

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Histamine H_1- and H_2-receptor-mediated gastric microcirculatory effects in the aetiology of stress ulceration in the rat stomach

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Summary. Stress produced severe mucosal ulcers, increased mucosal microcirculation and lowered mast cell counts in the glandular wall of rat stomachs. Mepyramine i.m. or metiamide i.p. effectively prevented both ulceration and microcirculatory changes but not stress-reduced mast cell counts.

Stomach wall mast cell degranulation with resulting mucosal microcirculatory changes are considered to play a dominant role in stress-induced gastric ulceration in rats[2-11]. Histamine H_1- or H_2-receptor antagonists decrease ulceration[2,3]. However, the mechanism of their anti-ulcer actions remains unclear. This study now examines the effects of histamine H_1- or H_2-receptor antagonism on gastric mucosal microcirculation and changes in mast cell counts in stressed rats.

Materials and methods. Male Sprague-Dawley rats (150–200 g), starved for 48 h beforehand, had free access to sucrose 8% in NaCl 0.2% w/v which was removed 1 h before starting experiments. Mepyramine maleate (M&B) Ltd) 6.3 or 25 mg/kg i.m. (expressed as the salt) or metiamide (SK&F Labs) 25 or 100 mg/kg i.p. were given, either separately or together, 0.5 h before stress. Similar volumes (2 ml/kg) of NaCl 0.9% w/v (saline) were given by the appropriate routes to the controls.

Animals to be stressed were put into close-fitting tubular restraint cages of wire mesh and exposed to 4 °C; controls were left in their starvation cages at room temperature (22±1 °C). After 2 h, all were killed by a sharp blow on the head. Stomachs, opened along the greater curvature, were examined for mucosal lesions with an illuminated magnifier (×3). Lesions were measured (mm) and their lengths expressed as an ulcer index[6]. The glandular segment of each stomach was processed for mast cell counts[6,9,12] and studies on mucosal microcirculation[7,9]. The number of metachromatically stained mast cells in 40 oil immersion fields (magnification × 1000) was counted in the mucosal, submucosal and muscle layers[6,7]. Microcirculatory volumes were determined by measuring the density of stained haemoglobin in the muscularis propia which acted as the tissue blank (TB), the upper third of the mucosa (SM) and the lower third of the mucosa (DM)[7,9]; differences between mean density readings (15 readings per area) indicated the relative blood volumes in the superficial (TB–SM) or deep (TB–DM) mucosa. Data were analysed using Student's t-test.

Results. All nonstressed groups showed low ulcer indices as occasional petechiae were found only in the glandular mucosa (table 1, A). Stress markedly increased the glandular ulcer index in saline-pretreated rats (table 1, B); the lesions appeared as haemorrhagic ulcers. Pretreatment with graded doses of mepyramine or metiamide progressively lowered the ulcer index in stressed animals. Combined lower doses tended to lessen ulcer severity further.

Stress significantly increased the microcirculatory blood volume only in the superficial gastric glandular mucosa of saline-pretreated rats (table 1, B). Mepyramine or metiamide pretreatment also reversed dose-dependently these stress-induced microcirculatory changes in the superficial mucosa. The relative blood volumes in the deep mucosa of nonstressed (table 1, A) or stressed (table 1, B) animals

pretreated with either saline or the drugs were not statistically different.

Mast cell counts in the glandular stomach wall of all non-stressed rats were statistically similar (table 2, A). Stress markedly reduced the cell counts in the 3 layers of the glandular wall of saline-pretreated animals (table 2, B). Neither mepyramine, metiamide nor their combined pretreatment affected stress-induced mast cell degranulation in the mucosal, submucosal and muscle layers.

Discussion. The findings that either mepyramine or metiamide dose-dependently prevented lesion formation and mucosal vasodilatation without influencing stress-reduced stomach mast cell counts not only substantiate the belief that histamine from gastric mast cells contributes to the ulceration[2-11,13], but also suggest that histamine H_1- and H_2-receptors both participate in the superficial mucosal microcirculatory changes involved in the pathophysiology of these ulcers[2-11]. Gastric H_1-receptor stimulation produces

Table 1. Effects of mepyramine or metiamide pretreatment on ulceration and microcirculatory changes in the gastric glandular mucosa of stressed rats

Pretreatment	Dose	No. of rats	Ulcer index (mm)	Relative blood volume (densitometer units) Superficial mucosa (TB-SM)	Deep mucosa (TB-DM)
A) Non-stressed groups (unrestrained at room temperature for 2 h)					
Saline (i.m.)	2 ml/kg	12	0.07 ± 0.04	12.94 ± 0.87	5.47 ± 0.67
Saline (i.p.)	2 ml/kg	12	0.06 ± 0.05	10.95 ± 0.77	4.89 ± 0.87
Saline (i.m.) + saline (i.p.)	2 ml/kg	12	0.04 ± 0.04	12.17 ± 0.91	5.09 ± 0.91
Mepyramine (i.m.)	6.3 mg/kg	12	0.05 ± 0.03	10.65 ± 0.80	3.30 ± 0.91
Mepyramine (i.m.)	25 mg/kg	12	0.08 ± 0.05	11.45 ± 0.82	3.75 ± 0.75
Metiamide (i.p.)	25 mg/kg	12	0.04 ± 0.02	10.34 ± 1.06	3.24 ± 0.76
Metiamide (i.p.)	100 mg/kg	12	0.06 ± 0.03	12.34 ± 0.64	3.03 ± 0.63
Mepyramine (i.m.) + metiamide (i.p.)	6.3 mg/kg 25 mg/kg	12	0.05 ± 0.02	10.06 ± 0.64	3.43 ± 0.47
B) Stressed groups (restrained at 4 °C for 2 h)					
Saline (i.m.)	2 ml/kg	14	7.58 ± 1.56[f]	19.86 ± 1.35[f]	5.49 ± 0.72
Saline (i.p.)	2 ml/kg	14	6.61 ± 0.91[f]	20.81 ± 1.17[f]	5.84 ± 0.74
Saline (i.m.) + saline (i.p.)	2 ml/kg	14	6.50 ± 0.88[f]	19.91 ± 1.00[f]	5.91 ± 0.63
Mepyramine (i.m.)	6.3 mg/kg	14	2.69 ± 0.40[b,f]	15.19 ± 1.09[a,e]	3.74 ± 0.74
Mepyramine (i.m.)	25 mg/kg	14	1.92 ± 0.27[b,f]	14.91 ± 0.88[b,e]	4.79 ± 0.74
Metiamide (i.p.)	25 mg/kg	14	1.31 ± 0.35[c,e]	13.72 ± 1.15[c,d]	5.08 ± 0.72
Metiamide (i.p.)	100 mg/kg	14	0.81 ± 0.29[c,d]	12.01 ± 1.16[c]	4.06 ± 0.62
Mepyramine (i.m.) + metiamide (i.p.)	6.3 mg/kg 25 mg/kg	14	0.51 ± 0.13[c,e]	12.64 ± 1.06[c]	4.15 ± 0.70

The values shown are means ± SEM. [a] $p < 0.02$; [b] $p < 0.01$; [c] $p < 0.001$; compared with its own control pretreated with saline by the same route. [d] $p < 0.05$; [e] $p < 0.01$; [f] $p < 0.001$; compared with its corresponding non-stressed group (A). TB = tissue blank, SM = superficial mucosa, DM = deep mucosa.

Table 2. Effects of mepyramine or metiamide pretreatment on changes in mast cell counts in the gastric glandular wall of stressed rats

Pretreatment	Dose	No. of rats	Mast cell count/40 o.i.f. Mucosa	Submucosa	Muscle
A) Non-stressed groups (unrestrained at room temperature for 2 h)					
Saline (i.m.)	2 ml/kg	12	97.3 ± 5.0	51.0 ± 1.5	11.5 ± 0.9
Saline (i.p.)	2 ml/kg	12	90.1 ± 9.7	51.9 ± 4.0	8.2 ± 1.9
Saline (i.m.) + saline (i.p.)	2 ml/kg	12	86.0 ± 9.7	45.7 ± 2.4	9.6 ± 1.9
Mepyramine (i.m.)	6.3 mg/kg	12	87.1 ± 9.1	50.7 ± 3.8	12.7 ± 0.8
Mepyramine (i.m.)	25 mg/kg	12	90.0 ± 8.8	55.4 ± 4.7	8.3 ± 0.7
Metiamide (i.p.)	25 mg/kg	12	85.8 ± 7.5	48.7 ± 4.0	9.9 ± 1.1
Metiamide (i.p.)	100 mg/kg	12	89.7 ± 7.8	49.1 ± 1.7	10.9 ± 0.4
Mepyramine (i.m.) + metiamide (i.p.)	6.3 mg/kg 25 mg/kg	12	80.5 ± 9.9	48.2 ± 2.1	14.1 ± 1.3
B) Stressed groups (restrained at 4 °C for 2 h)					
Saline (i.m.)	2 ml/kg	14	49.8 ± 4.9[d]	36.6 ± 4.4[c]	4.4 ± 1.4[d]
Saline (i.p.)	2 ml/kg	14	37.1 ± 3.0[d]	41.3 ± 3.4[a]	4.7 ± 0.8
Saline (i.m.) + saline (i.p.)	2 ml/kg	14	43.4 ± 7.0[d]	36.6 ± 2.3[b]	4.7 ± 1.2[a]
Mepyramine (i.m.)	6.3 mg/kg	14	43.8 ± 7.5[c]	34.7 ± 3.5[c]	6.8 ± 1.3[c]
Mepyramine (i.m.)	25 mg/kg	14	43.8 ± 5.6[d]	38.9 ± 3.2[c]	4.4 ± 0.7[d]
Metiamide (i.p.)	25 mg/kg	14	35.9 ± 3.4[d]	37.8 ± 3.3[a]	4.8 ± 1.4[b]
Metiamide (i.p.)	100 mg/kg	14	33.0 ± 4.1[d]	37.6 ± 4.0[a]	4.3 ± 1.1[d]
Mepyramine (i.m.) + metiamide (i.p.)	6.3 mg/kg 25 mg/kg	14	36.4 ± 6.2[d]	36.4 ± 2.9[c]	6.9 ± 2.4[a]

The values shown are means ± SEM. [a] $p < 0.05$; [b] $p < 0.02$; [c] $p < 0.01$; [d] $p < 0.001$; compared with its corresponding non-stressed group (A). o.i.f. = oil immersion field (× 1000).

mucosal vasodilatation[14], however it increases stomach contractions[15] which can lead to mucosal vascular engorgement[11]; both these effects are reflected by elevated microcirculatory blood volumes. Further investigations are therefore necessary to establish quantitatively their contributions to the increased microcirculatory volume, since blockade of either or both of these H_1- receptor effects could have been responsible for the observed action with mepyramine. The ability of H_2-receptor block to inhibit stress-induced mucosal vasodilatation is equally interesting, and may explain the antiulcer action of metiamide[3] and of doses of metiamide[16] or cimetidine[17] which do not influence gastric acid secretion. H_2-receptor stimulation in the stomach has so far been shown only to dilate mucosal microvessels[14,18], and to increase acid secretion[15] which does not contribute to stress ulceration in rats[19,20]. Mucosal vasodilatation is likely to be due to a direct vascular effect of released gastric histamine, and not secondary to increased histamine-mediated acid secretion[18], because the antiulcer action of H_2-receptor blockade occurs even when acid secretion is unaffected[16,17].

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Cyclic AMP is a likely mediator of ovulation in the tsetse fly[1]

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Summary. Ovulation in tsetse flies is normally induced by mating, but virgins can be stimulated to ovulate with an injection of dibutyryl cyclic AMP, cholera toxin (a cyclic AMP generator), or aminophylline (a phosphodiesterase inhibitor). Thus, elevation of cyclic AMP is a likely link in the events leading to ovulation.

Virgin females of many insect species produce mature eggs within their ovaries, but seldom are the eggs ovulated unless she has an opportunity to mate[2,3]. Such is the case in the tsetse fly, the vector of African sleeping sickness[4,5]. Successful mating is a prerequisite for ovulation, and the single egg matured in the ovary of a virgin tsetse is eventually resorbed if the female remains unmated. The act of mating triggers ovulation by stimulating the female's brain to release a neurosecretion that is conveyed by the blood to its target organ, the ovary[6]. A follicular plug at the base of the ovary ruptures and contractions of the oviduct propel the egg into the uterus. We suggest that cyclic AMP is a likely mediator of ovulation in tsetse flies since we find that ovulation can be induced in virgin females with dibutyryl cyclic AMP and other chemical agents that elevate levels of cyclic AMP.

Test compounds (cholera toxin was obtained from Schwarz/Mann and all other chemicals were from Sigma) were injected without anaesthesia into 12-day-old virgin female *Glossina morsitans morsitans* Westw. through the dorsal region of the thorax using a finely drawn, calibrated glass capillary. Flies of this age were selected to optimize the probability that the ovary would contain an egg that was fully mature but had not yet begun to be resorbed[6]. The incidence of ovulation was determined by dissecting the flies 24 h after treatment.

The 1st line of evidence implicating a role for cyclic AMP in tsetse ovulation comes from the effect elicited by an injection of dibutyryl cyclic AMP. As shown in the table, dibutyryl cyclic AMP (N^6, $O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphoric acid) caused 55% of the virgin females to ovulate. Cyclic AMP (adenosine 3′:5′-cyclic monophosphoric acid) and 8-bromo-cyclic GMP (8-bromoguanosine 3′:5′-cyclic monophosphoric acid) were ineffective. In several other organisms, dibutyryl cyclic AMP has also been found to be a more potent agent than cyclic

Effect of cyclic nucleotides, aminophylline, and cholera toxin on stimulating ovulation in 12-day-old virgin females of *Glossina morsitans*

Material injected	Number	Ovulation (%)
Distilled water (1 µl)	22	5
8-bromo cyclic GMP (25 µg)	18	0
Cyclic AMP (25 µg)	19	0
Dibutyryl cyclic AMP (25 µg)	22	55
Aminophylline (10 µg)	20	45
Aminophylline (10 µg) + dibutyryl cyclic AMP (25 µg)	17	53
Cholera toxin (0.5 µg)	21	33

An injection volume of 0.5 µl was used for cholera toxin and 1.0 µl for all other chemicals.