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MECHANISM OF ACTION OF CYSTEAMINE ON DUODENAL ALKALINE PHOSPHATASE

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Abstract—A single s.c. injection of cysteamine-HCl, a potent duodenal ulcerogen, drastically decreased duodenal alkaline phosphatase (DAP) activity in mucosal cells. The effect was detectable 4 hr after the injection of cysteamine, it was the most prominent at 12 hr and lasted less than 24 hr after the ulcerogen administration. Under the same experimental conditions cysteamine did not affect alkaline phosphatase activity in the liver. Furthermore, ethanolamine, the toxic but nonulcerogenic derivate of cysteamine, had no effect on the DAP activity if given in equimolar quantities. Thus, the effect of DAP activity seems to be a property of the duodenal ulcerogen, related only to the target organ, i.e. duodenal mucosa.

Since cimetidine administration and pylorus ligation, which both abolish HCl hypersecretion provoked by cysteamine, did not "protect" the enzyme from the depletion, it is hard to believe that gastric acid hypersecretion could be responsible for the enzyme depletion provoked by the ulcerogen. Dopamine metabolism disorders, induced by cysteamine administration in the brain and duodenal mucosa, are not causally related to DAP-depletion either.

Cysteamine most probably inhibits the DAP activity by three different mechanisms, by one acting directly on the enzyme molecules and by two others acting indirectly, through its adrenocorticolytic and stresogenic effects.

Intestinal alkaline phosphatase (ortophosphoric-monoester phosphohydrolases, EC 3.1.31) is a dimeric glycoprotein whose native Mr is 180 kDa [1]. It consists of two identical subunits (Mr 79,4 kDa) with two cooperative nonequivalent binding sites, one of high and the other of low substrate affinity [1, 2]. It is a metaloenzyme with 4 Zn^{2+} per mol of dimeric protein [3]. The enzyme requires Mg^{2+} [2, 4], while L-phenylalanine is known as a stereo- and organ-specific inhibitor of intestinal alkaline phosphatase [5, 6]. The alkaline phosphatase is located in the brush borders and in Golgi membranes of the mucosal cells along the intestine [7–9]. Physiological function of alkaline phosphatase, especially fraction originating from Golgi complex is not well known. The brush border localization suggests that the enzyme plays a certain role in the uptake of phosphate [2], in lipid absorption [5, 10], as well as in the transport of calcium in the intestine [9, 11, 12].

It has been shown recently by two groups of authors [13–15] that cysteamine-HCl, a potent duodenal ulcerogen, significantly decreases brush border duodenal alkaline phosphatase (DAP) activity. However, there is disagreement between the two groups of authors concerning the mechanisms of cysteamine action on DAP activity. Dieffenbach *et al.* [13] suggested that the depletion of the enzyme activity was provoked indirectly, through gastric acid hypersecretion induced by the ulcerogen. Contrary to this, Stiel *et al.* [15] reported that duodenal hyperacidity was not responsible for the selective alteration of DAP activity by the duodenal ulcerogen.

The results of the present study provide data which could help to elucidate the disagreement between the two groups of authors concerning the effect of gastric acid hypersecretion provoked by cysteamine, on the DAP depletion and to understand better the molecular mechanism of the ulcerogenic action of cysteamine at the level of its target tissue, i.e. duodenal mucosa.

MATERIALS AND METHODS

Animals and treatment. Wistar albino female rats (Torlak, Beograd) with the initial body weight of 150–200 g were used in the experiments. The rats were fasted 24 hr prior to each experiment and watered *ad libitum*.

Rats were divided into control and experimental groups. To study the time-dependent effect of cysteamine on DAP activity, groups of rats were injected subcutaneously (s.c.) with aqueous solution of cysteamine-HCl (Aldrich), 30 mg/100 g body weight and sacrificed 2, 4, 8, 12 and 24 hr later.

To find out which of the two functional groups in cysteamine (H_2N–CH_2–CH_2–SH) was responsible for the DAP depletion, a group of rats were injected s.c. with the equimolar quantities (16 mg/100 g) of its analog ethanolamine (Sigma) (H_2N–CH_2–CH_2–OH) and sacrificed 12 hr later.

To detect eventual *in vitro* effect of cysteamine and ethanolamine on DAP activity, homogenates of mucosal cells of proximal duodenum of intact rats were preincubated before the enzyme assay with different concentrations of cysteamine (0.25–3.5 μmol/ml) and ethanolamine (0.25–3.5 μmol/ml) for 15 min at 37°.

To study eventual influence of gastric hypersecretion provoked by cysteamine [14, 16] on DAP depletion, a group of rats were injected s.c. with cimetidine (Belomet, Podravka, Belupo), 20 mg/100 g b.w. 30 min before and 6 hr after cysteamine

(30 mg/100 g) administration. The other group of rats was submitted to pylorus ligation before cysteamine administration. Both groups of rats were sacrificed 12 hr after the cysteamine administration.

To study the effect of dopamine depletion provoked by cysteamine on DAP activity, shown recently by Szabo *et al.* [18, 21], an additional groups of rats were injected s.c. with dopamine-HCl (Farmakos, Prizren) 1 × 5 mg/100 g, with its agonist bromocriptin mesylate (Sandoz pharmaceuticals), 3 × 5 mg/100 g or 1 × 5 mg/100 g; and with its antagonist haloperidol (Krka, Novo Mesto) 5 × 0.1 mg/100 g. When the drugs were given 30 min before cysteamine (30 mg/100 g) the rats were sacrificed 12 hr after the ulcerogen injection.

To detect the contribution of adrenals to the DAP depletion after cysteamine administration, bilateral adrenalectomy was performed. The rats were then maintained on 0.9% sodium chloride drinking water until the 4th day when they received cysteamine (30 mg/100 g) and were sacrificed 12 hr later.

To study dose-dependent effect of propionitrile, groups of rats were injected s.c. with 2.5, 5 and 10 mg/100 g of the duodenal ulcerogena and sacrificed 12 hr later.

All rats were sacrificed by decapitation. Proximal duodenum was quickly removed and put on ice. Scrapings of mucosae, removed from the proximal segment of duodenum with a microscopic slide on a cold board, were homogenized (1 min at 1800 rev/min) in 0.1 M Tris–HCl (250 mM sucrose, 2 mM 1,4-dithioerythritol (Merck) (DTT)), pH 7.4 at 0–4°, using motor driven Teflon–glass homogenizer.

Determination of alkaline phosphatase activity. The enzyme activity was measured by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (pNPP) or Pi from Na-β-glycerophosphate (β-GP). The standard assay mixture for p-NPP-ase activity contained 50 mM bicarbonate buffer, pH 9.0; 1 mM DTT; 20 mM $MgCl_2$ and 20 mM pNPP in a volume of 1 ml. The reaction was carried out at 37° and terminated after 10 min adding 1 ml 1 M Na_2CO_3. The absorbance of the mixture was measured spectrophotometrically at 410 nm by using as a blank control lacking the enzyme. The extinction coefficient for p-nitrophenolate anion is $1.75 \times 10^4 \cdot M^{-1} \cdot cm^{-1}$.

When β-GP (20 mM) was used as a substrate, the incubation mixture contained 50 mM Tris–HCl buffer, pH 8.2, 1 mM DTT and 20 mM $MgCl_2$ in a volume of 1 ml. The incubation time was 15 min at 37°. The reaction was stopped by the addition of 1.5 ml 20% trichloroacetic acid. P_i released during the reaction was determined by the method of Briggs [19].

One unit of alkaline phosphatase was defined as the amount of the enzyme catalysing the release of 1 μM of *p*-nitrophenol per min from *p*-NPP, or 1 μM Pi per min from β-GP. Specific enzyme activity was defined as units/mg of protein.

Protein concentration was determined by the method of Bradford [20].

RESULTS

A. Duodenal alkaline phosphatase (DAP) activity in normal and in ulcerogens-treated rats

Figure 1 shows pH dependence of duodenal alkaline phosphatase with *p*-nitrophenyl phosphate (pNPP) and β-glycerophosphate (β-GP) as substrates. The enzyme exhibited the optimal activity at pH 8.2 with β-GP and a plateau of pH optimum between 8,2 and 9,0 (taken as optimal) with pNPP as substrates (Fig. 1). Judging by these data, β-

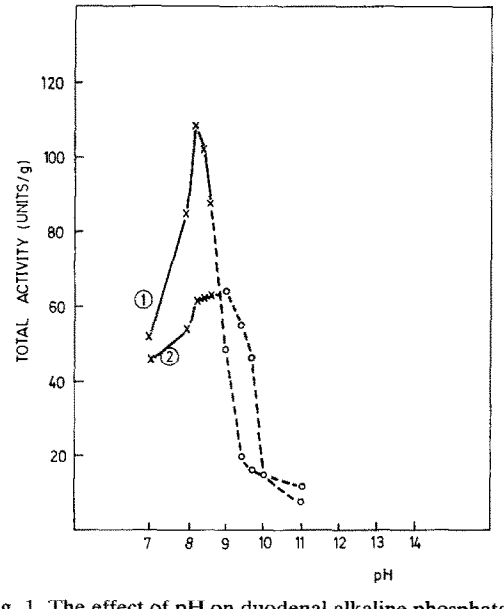

Fig. 1. The effect of pH on duodenal alkaline phosphatase (DAP) activity. (1). β-glycero phosphatase: —×—×— Tris–HCL buffer; ---O--O--Na_2CO_3–$NaHCO_3$ buffer. (2) *p*-Nitrophenyl phosphatase.

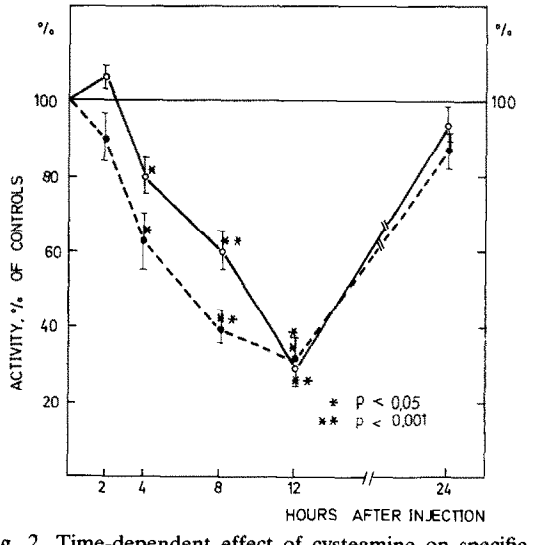

Fig. 2. Time-dependent effect of cysteamine on specific DAP activity under *in vivo* conditions. Cysteamine was given s.c., 30 mg/100 g and rats (seven in each group) were sacrificed at the indicated time after the injection. Specific activity, units/mg protein; —O—O—, *p*-nitrophenyl phosphatase; ---●---●--, β-glycero phosphatase. Statistically significant differences are indicated by asterisks; vertical bars = standard errors of mean values.

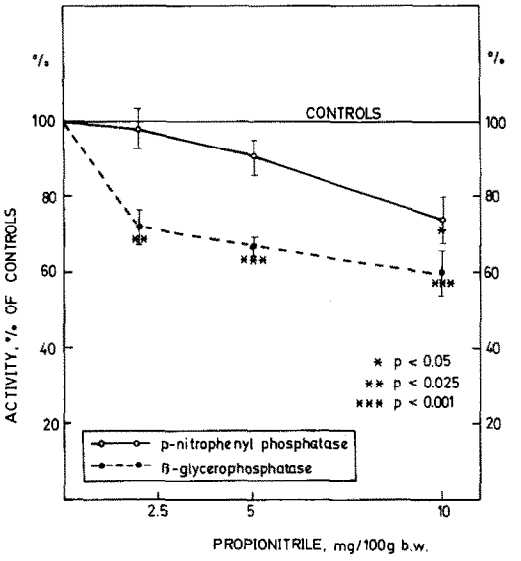

Fig. 3. Dose-dependent effect of propionitrile on specific DAP activity. The rats (five in each group) were injected s.c. with the indicated quantities of propionitrile and sacrificed 12 hr later. Statistically significant differences are indicated by asterisks; vertical bars = standard errors. Activity, units/mg protein.

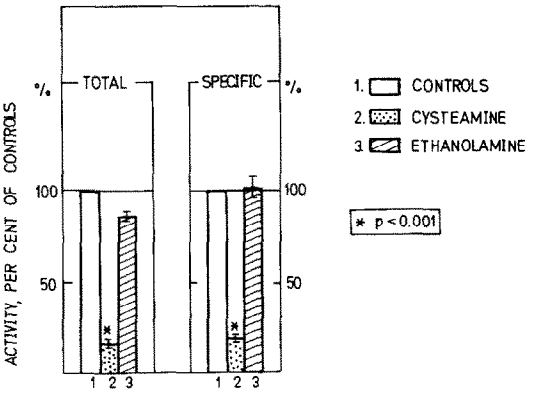

Fig. 4. Comparative effects of equimolar quantities of cysteamine and ethanolamine on basal DAP activity. Cysteamine (30 mg/100 g) and ethanolamine (16 mg/100 g) were injected s.c. and the rats (seven in each group) were sacrificed 12 hr later. Total activity, units/g. Specific activity, units/mg protein. Statistically significant differences are indicated by asterisks; vertical bars = standard errors of the mean value. *p*-Nitrophenyl phosphate was used as substrate.

GP-ase activity appears to be associated with an isoenzyme distinct from pNPP-ase.

Time-dependent effect of cysteamine on duodenal alkaline phosphatase activity under in vivo *conditions*

Figure 2 shows that a single dose of cysteamine-HCl (30 mg/100 g) induced significant and time-dependent depletion of the DAP activity with a maximum at 12 hr after the injection. The enzyme activity almost returned to the control value 24 hr after the injection (Fig. 2). Under the same experimental conditions cysteamine had no effect on the liver alkaline phosphatase activity (data not shown).

The dose-dependent effect of propionitrile on DAP activity

Propionitrile was shown to be a less potent duodenal ulcerogen than cysteamine [14]. Figure 3 shows that it was less effective in depleting DAP activity than cysteamine, as well (Fig. 3). It decreased the *p*-nitrophenyl phosphatase activity for about 26% (only after one s.c. injection of 10 mg/100 g b.w.), and *β*-glycero phosphatase activity, from 28 to 40% (after one s.c. injection of the ulcerogen from 2.5–10 mg/100 g b.w.) (Fig. 3).

B. The mechanism of DAP depletion provoked by cysteamine administration

In vivo *influence of ethanolamine on the basal DAP activity*

Ethanolamine (H_2N–CH_2–CH_2–OH) differs from cysteamine (H_2N–CH_2–CH_2–SH) in having —OH instead of —SH group. It is a toxic, but non-ulcerogenic analog of cysteamine. We compared the effect of both amines on the DAP activity under *in vivo* conditions. The results presented in Fig. 4 show that ethanolamine had no effect on the basal DAP activity when given s.c. in equimolar quantity with cysteamine.

Concentration-dependent effect of cysteamine and ethanolamine on DAP activity under in vitro *conditions*

When homogenate of duodenal mucosal cells was preincubated with different concentrations of cysteamine for 30 min, at 37°, the DAP activity was inhibited in a concentration-dependent manner with both substrates (Fig. 5). Equimolar quantities of

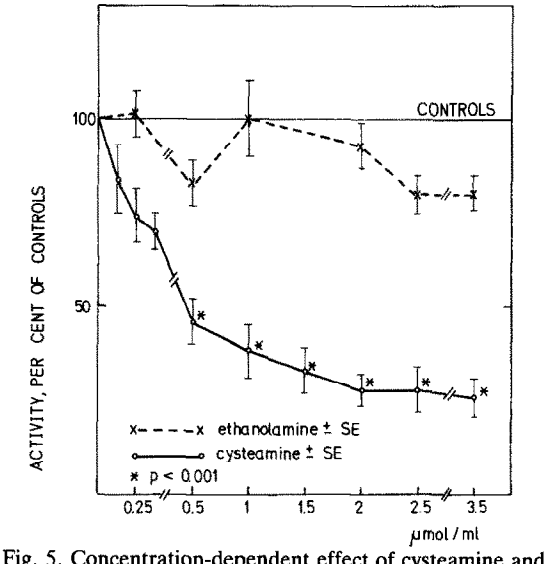

Fig. 5. Concentration-dependent effect of cysteamine and ethanolamine on DAP activity under *in vitro* conditions. *p*-Nitrophenyl phosphate was used as substrate. Each point represents the mean value of five experiments. Statistically significant differences are indicated by asterisks and standards errors of mean value by vertical bars. Activity, units/mg protein.

Table 1. The effect of pylorus ligation and cimetidine on basal and cysteamine-depleted duodenal alkaline phosphatase activity

	Protein (mg/g)		Alkaline phosphatase activity (Unit·mg protein^{-1}·min^{-1}·10^{-2}) p-nitrophenyl phosphatase		β-glycero phosphatase	
Controls	58.84 ± 1.84*	—	106.10 ± 5.07	—	155.53 ± 12.1	—
	(15)	(100)	(11)	(100)	(10)	(100)
Cysteamine	44.15 ± 2.96	<0.001†	22.31 ± 2.17	<0.001	28.80 ± 5.85	<0.001
(30 mg/100 g)	(10)	(75)	(8)	(21)	(8)	(19)
"Sham" operation	58.85 ± 2.67	NS	87.23 ± 7.47	<0.001	114.91 ± 18.35	<0.005
	(4)	(100)	(4)	(82)	(4)	(74)
Pylorus	61.10 ± 1.57	<0.025	90.01 ± 9.34	<0.005	111.85 ± 14.05	<0.001
ligation	(8)	(104)	(7)	(85)	(7)	(72)
Pylorus						
ligation	67.46 ± 3.14	<0.001	55.65 ± 4.89	<0.001	74.50 ± 8.15	<0.001
+ cysteamine	(6)	(114)	(5)	(52)	(5)	(48)
Cimetidine	54.07 ± 9.73	NS	95.77 ± 21.97	NS	117.89 ± 42.8	<0.025
(2 × 20 mg/100 g)	(5)	(92)	(5)	(90)	(6)	(76)
Cimetidine	49.34 ± 4.96	<0.001	49.08 ± 18.36	<0.001	77.80 ± 22.38	<0.001
+ cysteamine	(6)	(84)	(6)	(46)	(6)	(50)

* Mean values ± standard errors with (N) of rats.
† P value, Student's *t*-test, with (%) of changes.
NS = non-significant.

ethanolamine had no effect on DAP activity under the same experimental conditions (Fig. 5).

The effect of the gastric acid hypersecretion provoked by cysteamine on DAP activity

Enhanced gastric acid secretion and decrease in acid neutralization in proximal duodenum were found in rats treated with cysteamine and other duodenal ulcerogens [14, 16]. The contribution of the gastric acid hypersecretion to the depletion of the DAP activity after cysteamine administration was examined by the pretreatment of rats with histamine H_2-receptor antagonist cimetidine and by pylorus ligation.

(a) *The influence of cimetidine on the basal and cysteamine-depleted DAP activity*. Cimetidine alone induced a statistically significant decrease of the basal DAP activity (to 76% of controls) only with β-GP as a substrate (Table 1). Cysteamine decreased DAP activity to 21% of controls. When cimetidine was given once, 30 min prior to cysteamine, or twice, 30 min before and 5 hr after cysteamine, it had some incomplete "protective" effect. It attenuated the decrease of the enzyme activity induced by cysteamine from 21 to 46 per cent of controls (Table 1).

(b) *The effect of pylorus ligation on the basal and cysteamine-decreased DAP activity*. Table 1 shows that pylorus ligation and "sham" operation induced significant reduction in DAP activity, to 82 and 85% of control, respectively. However, when cysteamine was given after pylorus ligation the depletion of DAP activity was attenuated to 50% of controlls. The decrease was still statistically significant in comparison to the basal values found in the intact rats (Table 1).

The effect of dopamine depletion on DAP activity in cysteamine-treated rats

The disorders in dopamine metabolism after cysteamine administration to rats, followed by the decline of its concentration in the brain and duodenal mucosa, have been recently discovered [18, 21]. To study the involvement of the disorders in the decline of the DAP activity after cysteamine administration, separate groups of rats were injected with dopamine, its agonist bromocriptine and its antagonist haloperidol 30 min prior to the ulcerogen administration.

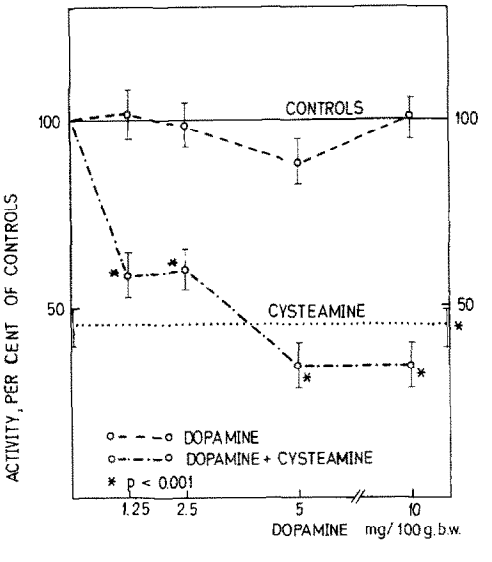

Fig. 6. Dose-dependent effect of dopamine on basal and cysteamine depleted DAP activity. Indicated doses of dopamine were injected s.c. 30 min prior to cysteamine (30 mg/100 g, s.c.). The rats (seven in each group) were sacrificed 12 hr after cysteamine. The enzyme activity was tested with *p*-nitrophenyl phosphate as substrate. Basal activity was taken as control (100%). Statistically significant differences are indicated by asterisks and standard errors of the mean values by vertical bars.

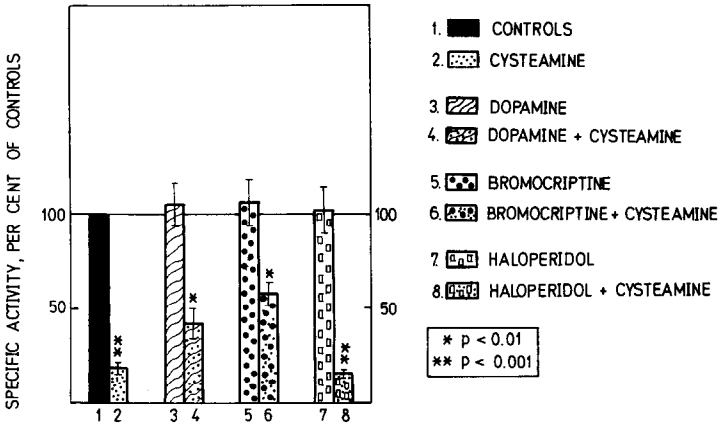

Fig. 7. The influences of dopamine, bromocriptine and haloperidol on basal and cysteamine-decreased DAP activity. Cysteamine (30 mg/100 g), dopamine (5 mg/100 g), bromocriptine (5 mg/100 g) and haloperidol (5 × 0.1 mg/100 g) were given s.c. to the rats (5–7 in each group) who were sacrificed 12 hr later. When given together dopamine, bromocriptine and haloperidol were injected 30 min prior to cysteamine and the rats were sacrificed 12 hr after the injection of the ulcerogen. Activity (units/mg proteins) was tested with *p*-nitrophenyl phosphate as substrate. Statistically significant differences are indicated with the asterisk and standard errors of the mean value with the vertical bars.

As seen from Figs. 6 and 7 dopamine had no effect on the basal DAP activity. Cysteamine (30 mg/100 g, s.c.), decreased the basal DAP activity to 20% (Fig. 7) and 45% (Fig. 6) of controls. When rats were injected with dopamine (1–10 mg/100) 30 min before cysteamine, the depletion of DAP activity was poorly attenuated (Figs 6 and 7). Figure 7 shows in addition that bromocriptine, a dopamine agonist, has exactly the same effect as dopamine on both the basal (control) and cysteamine-depleted DAP activity. Dopamine antagonist haloperidol (5 × 0.1 mg/100 g s.c. or 1 × 1 mg/100 g, s.c.) did not change the pNPP-ase activity. Pretreatment of rats with haloperidol 30 min prior to cysteamine, had no effect on the level of the enzyme depletion provoked by the administration of the ulcerogen (Fig. 7).

The effect of adrenalectomy on the basal and cysteamine-depleted DAP activity

Figure 8 shows that adrenalectomy alone decreased significantly DAP activity as compared to intact controls. The effect of adrenalectomy was even additive with the cysteamine-induced decrease of DAP activity (Fig. 8).

DISCUSSION

Our results confirmed previously reported data [13–16] showing that cysteamine drastically decreased DAP activity with the most prominent effect at 12 hr after a single injection (Fig. 2). The enzyme activity returned to the basal value 24 hr

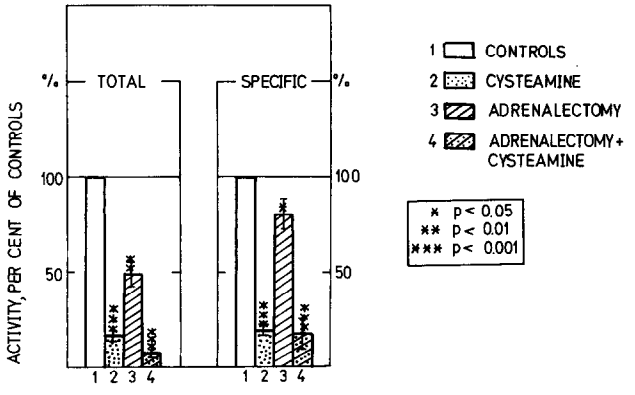

Fig. 8. Influence of cysteamine on DAP activity in adrenalectomized rats. Adrenalecomized (bilateral) rats were maintained on 0.9% NaCl-drinking water 4 days before cysteamine (30 mg/100 g, s.c.) administration. The rats were sacrificed 12 hr after the ulcerogen administration. Total activity, units/g; Specific activity, units/mg protein. The activity was tested with *p*-nitrophenyl phosphate as substrate. Statistically significant differences are indicated by asterisks; vertical bars = standard errors of mean values.

after the cysteamine injection (Fig. 2). The duodenal ulcerogen had no effect on the alkaline phosphatase activity in the liver (data not shown). Thus, the effect on DAP activity seems to be related only to the target organ of the ulcerogen, i.e. duodenal mucosa. The results with ethanolamine, a toxic non-ulcerogenic structural analog of cysteamine, further reinforces the specificity of this enzyme effect and indicate that —SH groups in cysteamine molecule are responsible for the DAP depletion (Figs 4 and 5).

When the homogenate of the duodenal mucosal cells was preincubated with different concentrations of cysteamine under *in vitro* conditions, before the enzyme assay, the DAP activity was inhibited in a concentration-dependent manner as well (Fig. 5). The results could indicate the direct effect of cysteamine on DAP molecules.

To assess the contribution of the gastric acid hypersecretion and the decrease in acid neutralization in proximal duodenum found by Szabo *et al.* [14, 22] and Kirkegaard *et al.* [16] to the depletion of DAP activity, we injected separate groups of rats with cimetidine or ligated pylorus 30 min before cysteamine administration. The results of these experiments indicated that neither chemical nor mechanical prevention of HCl disposal to duodenum were able to completely prevent the decrease of DAP activity found 12 hr after the cysteamine administration (Table 1). The results are in agreement with the data of Stiel *et al.* [15] and Kirkegaard *et al.* [16], who found that cysteamine exhibited maximal effect on gastric acid hypersecretion 5 hr after administration of the ulcerogen with the return to control value the next 10–11 hr, the time at which, in our experiments, the effect of the ulcerogen on DAP depletion was the most prominent (Fig. 2). The appearance of these two effects at different times after the ulcerogen administration indicate that they need not be causally related.

Our results are, however, in apparent disagreement with the finding of Dieffenbach *et al.* [13]. The authors found that cimetidine pretreatment and pylorus ligation protected completely DAP activity from the decrease provoked by cysteamine administration [13]. On the basis of those results they concluded that the duodenal ulcerogen was probably not directly responsible for the DAP depletion contrary to our conclusion after *in vitro* experiments, which showed that cysteamine could have a direct inhibitory effect on the DAP molecules (Fig. 5). Our results obtained in terms of the time-dependence of the cysteamine effect on the DAP activity (Fig. 2), suggest that Dieffenbach *et al.* [13] chose inappropriate time after the injection of the ulcerogen to test the possible protective effect of cimetidine or pylorus ligation on DAP activity. They evaluated the protective effect of both treatments 4 hr after the injection of cysteamine, when the acid hypersecretion is maximal [16], but according to our experiments, the DAP depletion was not always statistically significant (Fig. 2). It is better to test the protective effect of cimetidine and pylorus ligation 12 hr after cysteamine administration when DAP depletion was the most prominent (Fig. 2).

The results presented in Figs 6 and 7 show that dopamine metabolism disorders, found by Szabo *et al.* [18, 21] in the brain and in duodenal mucosal after cysteamine administration, are not causaly related to the DAP depletion in mucosal cell of duodenum. On the other hand, we have shown recently that protein phosphatase depletion, provoked by cysteamine administration, was causally related to the dopamine metabolism disorders [23, 24]. Dopamine and bromocriptine administration, 30 min before cysteamine prevented the enzyme from the depletion. However, the decrease of the protein phosphatase activity was even more profound in the rats which received the dopamine antagonist haloperidol prior to cysteamine [24]. The results indicate that the early molecular changes in preulcerogenic mucosal cells of duodenum could be of pluricausal origin and therefore triggered by different mechanisms. These results also reinforce the concept which recognizes duodenal ulcer as a multifactorial disease.

Table 1 and Fig. 8 show that the DAP activity was very sensitive to various interventions. Cimetidine administration, pylorus ligation, adrenalectomy and "sham" operation alone induced a decrease in DAP activity which reached statistical significance. These changes in DAP activity seem to be of nonspecific stresogenic origin. Cysteamine has been recently recognized as a stressor, because of the transient elevation it produces in plasma corticosterone, glucagon, insulin and glucose levels [17]. Further, it is known that cysteamine causes adrenocortical necroses [25]. An increased release of corticosteroids into circulation precedes the macroscopic necrotic lesion of the adrenals [17].

The relationship between the adrenocortical activity and DAP activity has already been established [26–28]. Hugon *et al.* [26] reported diurnal variations in the alkaline phosphatase activity of duodenum in mice that were negatively correlated with the diurnal variations in the concentration of circulating steroids. The activity of intestinal alkaline phosphatase in some animals species was reduced by adrenalectomy [27] and in others (adult rats) after cortisol administration [28]. All these results indicate that some relationship could exist between DAP depletion and the adrenocorticol lesions which develop in rats after cysteamine administration in addition to duodenal ulcer. It is possible that the DAP depletion after cysteamine administration could be provoked at least in part by both the chemical adrenalectomy, induced by corticolysis [25], and by its influence on the early release of corticosteroids into circulation [17]. The milder DAP depletion provoked by proprionitrile administration, which does not provoke adrenal cortical necroses but only duodenal ulcer [22], speaks in favour of this possibility (Fig. 3).

In conclusion we could say that cysteamine may inhibit the activity of DAP molecules by direct effect on the enzyme molecules, as well as indirectly, by its effect on adrenals and other endocrine glands. Since cimetidine administration and pylorus ligation, which abolished HCl hypersecretion provoked by cysteamine [14, 15], did not "protect" completely the enzyme from the depletion, it is hard to believe that gastric acid hypersection could be responsible for the enzyme depletion provoked by cysteamine.

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