the distribution of cells among different phases of the cell cycle (as calculated from their DNA content) was not greatly affected during the first 9 h of drug exposure[18]. These cyclic peptides thus appear not to act on a process directly involved in entry into or preparation for cell division. Microscopic observation of cells treated at higher drug concentrations revealed vacuolisation, enlargement, and, starting at 9 h, a progressive destruction of the cells and a decrease in the mitotic count. Cyl-2 has not yet been tested against mammalian cells because of insufficient toxin supplies.

Chlamydocin and HC-toxin have several biological and chemical similarities, besides both being cyclic tetrapeptides and containing Aoe. Both require an intact epoxide for activity[9,19]. Both affect growth of mouse mastocytoma cells and *hmhm* maize at low concentrations. Neither compound causes rapid death of mammalian cells or nondividing leaf mesophyll protoplasts[20]. Both have the same conformation in chloroform, with four *transoid* amide bonds and a bis-γ-turn[7,21]; this may be significant because the correct conformation is required for biological activity of tentoxin, another phytotoxic cyclic tetrapeptide[22]. (However, a synthetic analog of Cyl-2, cyclo(D-O-methylTyr-L-Ile-L-Pro-L-Leu), has a trans-trans-cis-trans conformation, so Cyl-2 might differ in this respect from HC-toxin and chlamydocin[23]). Because of these similarities, HC-toxin and chlamydocin, and perhaps also Cyl-2, may have the same cellular site of action, a site which would be common to animal and plant cells.

One or both of two structural differences must be responsible for the biological differences reported here between HC-toxin and chlamydocin. Whereas chlamydocin and Cyl-2 contain the amino acid sequence Pro-Aoe or Pip-Aoe, HC-toxin has the reverse sequence Aoe-Pro (figure). Chlamydocin and Cyl-2 also contain an aromatic amino acid instead of alanine and are thus larger and more hydrophobic.

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5 Abbreviations: Aib = aminoisobutyric acid; Ala = alanine; Ile = isoleucine; Leu = leucine; Phe = phenylalanine; Pip = pipecolic acid; Pro = proline; Tyr = tyrosine.
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Sodium deoxycholate promotes the absorption of heparin administered orally, probably by acting on gastrointestinal mucosa, in rats

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Summary. Sodium deoxycholate (DOC), selected as a promoter of gastrointestinal absorption of heparin, was administered orally to rats, followed, at increasing intervals, by heparin. Maximal plasma clearing activity (PC) was obtained with a 60-min interval, though PC was still elicited after 24 h, suggesting that DOC acts on the gastrointestinal mucosa. Inhibition of blood coagulation was also observed after oral heparin. The suggestion that DOC increases heparin absorption is supported by increased plasma levels of heparin. No signs of several gastrointestinal damage were seen.
Key words. Deoxycholic acid; heparin; enteral absorption.

It is widely accepted that heparin cannot be absorbed unaltered after oral administration. In fact, when introduced by this route it fails to evoke either blood plasma clearing activity (PC) or inhibition of blood coagulation, the two most typical pharmacological effects elicited by its parenteral injection, and this failure cannot be ascribed to enteral inactivation, either spontaneous[1] or enzymatic. Because of the interest of pharmaceutical oral heparin for therapeutic purposes, various procedures have been devised and tested in order to obtain PC and the anticoagulant effect after enteral application of heparin. In particular, the enteral coadministration of heparin and certain surfactants or bile acids[1-6] has been exploited.

It has previously been shown that pharmacologically active quantities of heparin are absorbed into the systemic circulation after intraesophageal administration[5], injection into the small intestine[3,7] and insertion in the colon[8], when administered together with certain bile salts. It has also been demonstrated that bile salts greatly improve enteral absorption of a number of chemically unrelated drugs[9]. However this evidence did not allow any firm inference about whether sodium deoxycholate (DOC), in particular, and surfactants in general, acted on the heparin molecule or affected the gastrointestinal mucosa, although the bulk of the evidence rather favors an influence on the enteral mucous membrane[10,11]. In order to obtain some in-

direct evidence on this question, and with a view to the possible clinical interest of the experimental model, we investigated whether the oral administration of heparin remained effective after oral DOC pretreatment.

Methods. Female Wistar rats, 180–200 g b.wt (S. Morini Farm, S. Polo d'Enza, Reggio Emilia, Italy) fasting for 12–14 h were used throughout. They were housed in a quiet climatized (22 ± 1 °C, 60% humidity) room with a natural light-dark cycle.

Drugs, dissolved in distilled water, were administered to urethane anesthetized (1.25 g/kg, i.p.) animals through an esophageal tube (hereafter termed oral administration) at a fixed solution volume of 5 ml/kg. Some groups of animals received, in a tail vein, heparin dissolved in saline at a fixed volume of 1 ml/kg, as control. In all cases blood samples were taken by means of heart puncture 30 and 60 min after i.v. and oral heparin respectively.

When DOC pretreatment preceded heparin treatment by more then 8 h, heparin was administered to conscious animals, blood samples being taken under light ether anesthesia.

2.35 ml of blood were collected in centrifuge tubes containing 0.14 ml of a 20% solution of citric acid trisodium salt in distilled water, and plasma was immediately separated by centrifugation in a refrigerated centrifuge (2200 × g for 15 min). PC was then assessed by measuring the decrease in OD of a mixture of plasma and a fat emulsion (lipostrate CB A grade, 0.20% in distilled water), as suggested by Grossman[12] with minor modifications[13]. In brief, 0.5 ml of the aqueous lipostrate emulsion were added to 1 ml of plasma and thoroughly mixed. 30 sec exactly after addition of lipostrate emulsion (time 0) the sample OD at 650 μm was recorded. Thereafter the mixture was incubated at 25 °C for 60 min and the OD measured again. The percentage OD variation was taken as PC.

Blood clotting time was deduced by verifying, every 30 sec up to 15 min, the time that elapsed, under standard conditions, between the deposition of 0.05 ml of blood into a watch glass and the formation of the first fibrin filament as suggested by Gigante and Raggio-Guarnaschelli[14].

Heparin-like material present in blood plasma was measured by the Boehringer (Mannheim, FRG) 'heparin low dose test' diluting the plasma where necessary with distilled water. Plasma was obtained by mixing 1 part of 0.11 molar sodium citrate solution into 9 parts of freshly collected blood and immediately centrifuging at 2000 × g for 10 min in a refrigerated centrifuge.

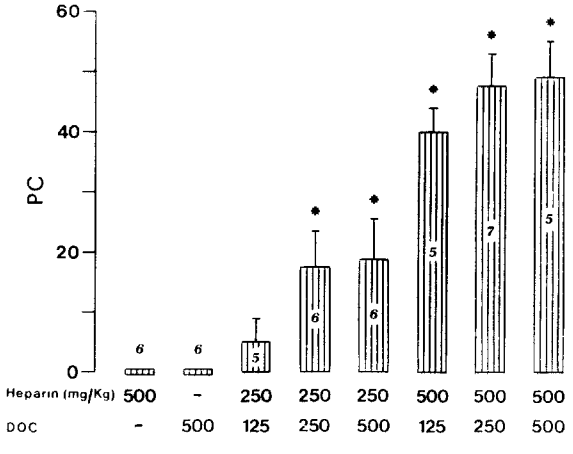

Figure 1. PC of rat blood plasma induced by oral heparin sodium plus DOC. Dose-effect relationship. Rats received orally 250 and 500 mg/kg of heparin together with 125–500 mg/kg of DOC. Histogram heights indicate mean PC (±SEM); number over or within histograms shows the number of rats used. * $p < 0.02$, at least, versus value for oral heparin alone (Student's t-test).

Heparin sodium was supplied by Prodotti Gianni (Milan, Italy: lot 965/734, 177 IU/mg, USP XX) and sodium deoxycholate from Oxoid Italia (Milan, Italy).

Results. Simultaneous oral administration of DOC (at least 250 mg/kg for a significant effect) and heparin sodium evokes PC, its intensity depending on a) the heparin dose (fig. 1) and b) the time of blood sampling (data not shown). On the basis of these results and of those of previous experiments showing that after the oral coadministration of heparin and Brij 56 (a nonionic surfactant promoting the gastroenteral absorption of heparin in rats)[15] the optimum time for blood sampling is 60 min after treatment, the influence of the interval between the separate oral administration of DOC and heparin was assessed in rats receiving 500 mg/kg of both drugs, sampling blood 60 min after heparin administration. Figure 2 shows that PC peaked when heparin administration followed DOC administration after 60 min. When heparin administration was postponed by intervals greater than this, PC was reduced further and further, being however still detectable after a 24-h interval. Further, figure 2 also shows that PC caused by oral heparin administered to rats pretreated 60 min previously with oral DOC was greater than that evoked by the simultaneous oral administration of the two drugs ($p < 0.001$, Student's t-test). At the same time oral DOC (500 mg/kg) did not influence PC induced by i.v. administration of 1 mg/kg of heparin: in fact simple heparin injection caused PC (70.15 ± 2.18, $n = 6$) in control rats not statisticly different from that found in rats given oral DOC 60 min before heparin (69.11 ± 3.12, $n = 6$, $p > 0.05$, Student's t-test).

Adequate heparin absorption into the systemic circulation inhibits blood coagulation besides inducing PC. Although it has been suggested that heparin is more potent as a PC inducer than as a blood coagulation inhibitor[8], we investigated whether oral heparin given to DOC-pretreated rats also affected blood clotting time. Accordingly, DOC (500 mg/kg) was administered orally to rats receiving heparin (500 mg/kg) by the same route 60 min later. Control animals, instead of DOC and/or heparin, received only water. A further control was also intro-

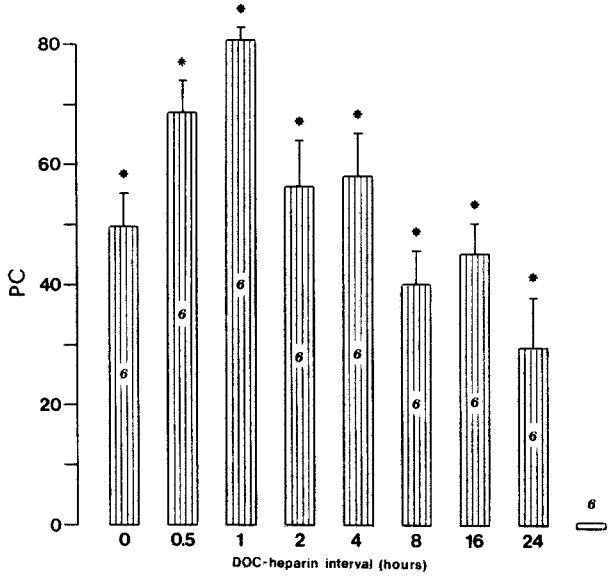

Figure 2. Ability of heparin to induce PC also when administered to rats a considerable number of hours after DOC. 500 mg/kg of DOC were administered orally to fasting rats and, after 0–24 h, the same amount of heparin. Histogram heights indicate mean PC (±SEM); number over or within histograms shows the number of rats used; the numbers under the histograms the DOC-heparin interval in h.
* $p < 0.01$, at least, versus value for oral heparin alone (open histogram) (Dunnett's test for multiple comparison with a control).

duced: heparin (1 mg/kg) injected i.v., which gave a blood level of heparin comparable to that observed after oral heparin in DOC pretreated rats. Figure 3 shows that oral DOC pretreatment enables oral heparin to inhibit blood coagulation, whereas it does not influence the prolongation of clotting time induced by i.v. heparin.

Evaluation of heparin-like material gave relatively high values with blood plasma of rats pretreated orally with DOC (500 mg/kg) and receiving heparin (500 mg/kg) by the same route 60 min after, but not with blood plasma of rats receiving only heparin of DOC (fig. 4).

It must be stressed, finally, that on no occasion were there overt signs of gastrointestinal damage such as diarrhea, hemorrhages, etc., to which the gastrointestinal absorption of heparin could be attributed.

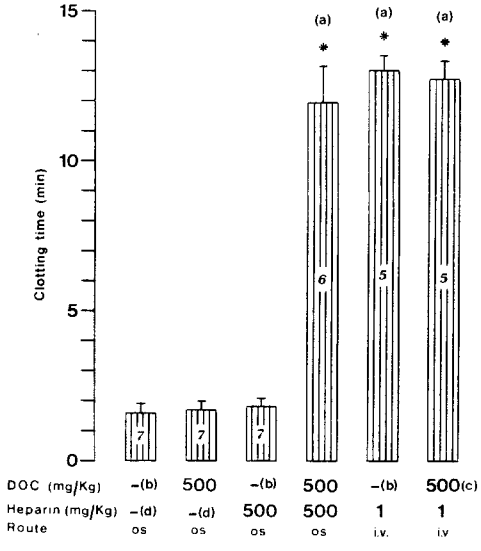

Figure 3. Prolongation of blood clotting time after oral administration of heparin in DOC-pretreated rats. Comparison with i.v. heparin. DOC was given orally to rats: after 60 min they received heparin by the same route or i.v. a: In two rats clotting time was greater than 15 min. b: Oral water instead of DOC. c: Oral DOC. d: Oral water instead of heparin. Histogram heights indicate mean (±SEM) clotting time, number within each histogram the number of rats. * $p < 0.001$ versus value for oral water alone (Dunnett's test for multiple comparison with a control).

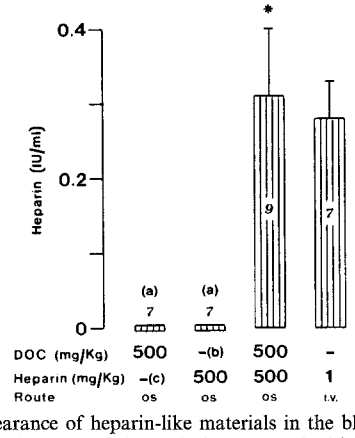

Figure 4. Appearance of heparin-like materials in the blood plasma of rats pretreated with DOC and 60 min later treated with heparin orally. Comparison with i.v. heparin. a: Below lower limit of method sensitivity. b: Water instead of DOC. c: Water instead of heparin. Histogram heights indicate mean (±SEM) heparin IU/ml, number over or within each histogram the number of rats. * $p > 0.05$ versus value for heparin i.v. (Student's t-test).

Discussion. The evidence reported suggests that DOC enables oral heparin to produce PC and prolongation of bloof clotting time by acting on the gastrointestinal mucous barrier rather than on the heparin molecule itself. Indirect support for such a view is afforded by Touitou's et al.[16] experiments on the hypoglycaemic effect, in diabetic rats, of intrajejunal administration of insulin performed 30 min after the intrajejunal injection of cetomacrogol 1000 (a nonionic surfactant also promoting enteral heparin absorption)[6], and also by Davis' et al. experiments[10]. These workers introduced POE-24 cholesteryl ether (another nonionic surfactant) into a canine gastric pouch for 5 to 60 min, rinsed the pouch cavity several times and then applied a solution of cephalotin without surfactant: a hyperabsorption state appeared which was the higher, the longer the exposure to the surfactant. In our experimental model the DOC-induced hyperabsorptive state persists for 24 h at least. Thus, the alteration of enteral mucosa caused by DOC differs from that produced by salicylates, for sodium salicylate and 5-methoxysalicylate a) are maximally effective promoters of rectal absorption when given simultaneously with insulin and b) their effect disappears quickly[17,18].

Evidence to hand does not allow us to define the precise mechanism through which DOC promotes the gastrointestinal absorption of heparin. It has been recognized that surfactants increase the enteral absorption of per se unadsorbable drugs, including heparin, by a certain disruption – as yet not precisely identified and occurring to a greater or lesser extent – of the histological, biochemical or functional features of the mucosal membrane[4,7,8,11,17,19–23]. However, a) not all surfactants are able to promote heparin absorption from the gastrointestinal tract, and b) there is no positive correlation between surfactant potency and promotion of enteral absorption, suggesting a selective rather than aspecific mechanism of action.

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