A Selective De-Heparinizer Filter Made of New Cross-Linked Polymers of a Poly-Amido-Amine Structure

Heparin is a well-known anticoagulant agent[1]. One of its main uses is the prevention of the thrombogenic action of extracorporeal apparatus employed, for example, in haemodialysis or cardiovascular surgery.

In both cases it is essential to control heparin's anticoagulant effect. Protamine is at present the only widely used heparin antagonist. This antagonist is, however, not devoid of risks[2-5].

In a previous investigation we found that a soluble polymer containing tertiary amino groups is able to interact with heparin[6]. Here we report the results obtained with two purpose-tailored cross-linked polymers. These proved able to interact specifically with heparin both in vitro and in vivo, without any undesirable effect on blood composition or function: in particular they do not alter the blood clotting mechanism[7].

Materials. The two substances we used, coded as E_2 and E_3, are polymers containing amido- and tertiary amino-groups in the main chain and carboxyl groups in the side chain. Their basic formulae are reported in Figure 1.

They can be obtained in a linear, water-soluble form by polyaddition through hydrogen transfer of an equimolecular mixture of *as* -N, N-dimethylethylenediamine and glycine (E_2) or 2,3-*trans*-piperazine-dicarboxylic acid (E_3), to 1,4-*bis*-acryloylpiperazine in water solution[8]. Crosslinked E_2 and E_3 suitable for de-heparinizer filters were obtained by substituting in the polymerization process, a part of the aminic monomers for ethylene diamine, which act as a tetrafunctional monomer.

Methods. The heparin-adsorbing capacity of the polymers was studied in vitro in water, plasma and citrated blood using the polymer gels in the form of small particles, mainly ranging between 5–50 μm ∅, and large particles of around 150–500 μm ∅. Water plasma, or blood containing heparin[9] at different concentrations (c = 10, 10^2, 2.10^2 or 10^4 μg/ml), was incubated or passed through a given amount of crosslinked polymer particles retained by a porous diaphragm in a plastic container. The adsorbing capacity was evaluated by indirect dosage of the residual heparin found in the filtered solutions, by using coagulative tests based on thrombine time (TT)[10] and recalcification time (RT)[11]. The adsorbing capacity was studied in relation to the size of the particles, heparin concentration, temperature and the time the solutions were in contact with the particles.

The heparin adsorbing capacity from blood was determined a) in vitro and b) in vivo. a) The in vitro experiments were performed at low speed by dropping blood by gravity through a container filled of particles acting as a

Table I. Relationship between the flow rate and the heparin-adsorbing capacity of E_2R and E_3R in water solution

Polymer[a]	Flow rate	Heparin adsorbed (%)
E_2R	80 ml/min	70
	60 ml/min	80
	40 ml/min	90
E_3R	80 ml/min	80
	60 ml/min	85
	40 ml/min	90–95

[a] Crosslinked by 30 moles/100 ml of ethylene diamine (based on the total amount of aminic monomers).

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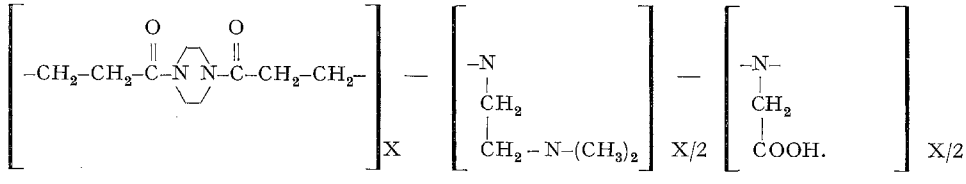

Fig. 1a) Formula of the polymer E_2

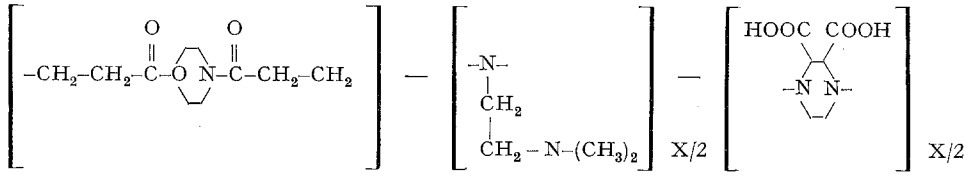

Fig. 1b) Formula of the polymer E_3

Table II. Partial thromboplatin time (PTT), prothrombin time (PT) and thrombin time of heparinized or nonheparinized blood samples filtered through crosslinked polymer E_2R

Citrated human blood	Treatment	PTT	PT	TT	Adsorbed heparin (%)
No heparin added	Control	46″	15″	9″	—
	filtered at 2 ml/min	45″	15″	8″	—
10 μg/ml heparin added	control	2′50″	36″	1′35″	—
	filtered at 2 ml/min	1′20″	15″	19″	85
	filtered at 8 ml/min	1′40″	18″	42″	65

filter. b) The in vivo experiments were performed on anesthetized dogs. A container was filled with 0.5 g of E_2 (0.1–0.5 mm ∅) retained by a suitable net. This filter was inserted in an extracorporeal artero-venous circuit; blood flow was maintained by Watson-Marlow rolling pump. Heparin was administered regionally by continuous dropping of heparin solution upstream of the filter.

The investigations to asses the possible effects on the blood clotting mechanism and blood composition were performed by evaluating certain parameters on plasma or blood samples which had been previously incubated or passed through the filter described.

These parameters were: a) the degree of haemolysis; b) sero-and lipo-protein electophoretic tracing; c) investigation of blood clotting factors by the dosage of fibrinogen and by evaluation of TT, RT, partial thromboplastin time (PTT)[11] and prothrombine time (PT). Platelets count was also performed according to the method described by BRECHER and CRONKITE[12].

Results. Our investigation showed that: 1. Both crosslinked polymers are capable of adsorbing heparin, as well at room temperature and 37°C, to such an extent that it is impossible to wash it out, at any rate in the interval of pH between 6 and 8.

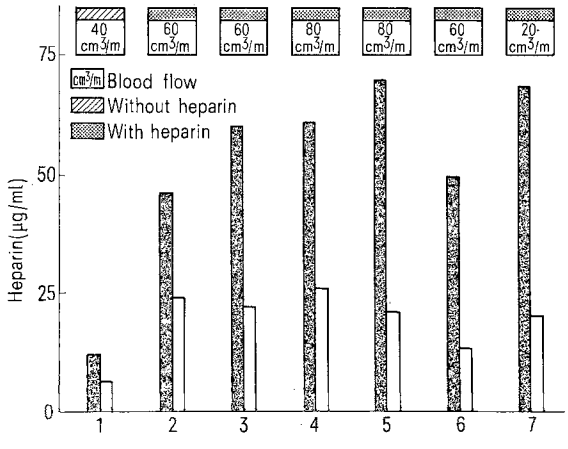

Fig. 2. Repeated samples of blood in a extracorporeal circuit, regionally perfused with heparin, working at different speed. The numbers indicate each heparine dosage done contemporary upstream (■) and downstream (□) of the E_2R inserted filter.

2. Time of contact as an expression of flow speed through the filter is important since the percentage of heparin adsorbed by the filter is in direct relation to the speed of flow of the heparin water solution as is shown in Table I.

3. Particle size plays an important role in the percentage value of bonded heparin. In the case of long time contact (10–15 min) and small amounts of polymer, the percentage of heparin captured from an aqueous solution by the small particles was 85–95%, while the large particles captured 50–80%, apparently irrespective of the heparin concentrations used. In the most favourable conditions the polymers are able to adsorb up to an amount of heparin equal to their own weight.

4. Both crosslinked polymers also act as good heparin adsorbers in blood. The results obtained with E_2R in vitro and in vivo are reported in the Table II and in Figure 2, respectively. The filters used in the two sets of experiment were different in size, number and distribution of the particles of the polymer. Hence the efficiency in vivo seems to be rather higher. Similar results were obtained with the polymer E_3R.

5. Neither of the crosslinked polymers in the conditions we used has any haemolytic action on RBC, nor do they alter RT, PT, PTT, TT or fibrinogen content. These results were also observed in an experiment of blood filtration at the low speed of 2 ml per min (Table II).

6. Neither of the polymers apparently interfere with the macromolecules normally present in the blood. In fact, electrophoretic tracings made on a sample of 1 ml of serum that had been in contact with 100 mg of the polymers did not show any modification either in the absolute or the relative value of the fraction investigated (albumine, α_1, α_2, β, and γ globuline and α and β lipoprotein).

7. Platelets count was not affected by the polymers under the following 2 experimental conditions A) after filtration of fresh human blood with added EDTA and B) after filtration of pure human blood just withdrawn from the vein.

Conclusions. In conclusion the polymers proved to be selective heparin adsorbers, devoid of any undesirable effect on the blood, at least in the flowing conditions we used. Their biological effects and physical and chemical properties lead us to visualize their being used to make molecular filters capable of de-heparinizing blood. Application of the filter in clinical apparatus can probably be

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envisaged, provided that the technological and physical problems are properly solved[13].

Riassunto. Due polimeri reticolati di nuova sintesi, contenenti gruppi amminici terziari, sono in grado di assorbire selettivamente eparina dal sangue. Essi non sono emolitici e non interferiscono con i fattori della coagulazione.

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The in vitro Effects of Several Progestogens, Estrogens and Non-Steroidal Compounds with Estrogenic Activity on Adenosine Diphosphate Induced Guinea-Pig Platelet Aggregation

The increased use of oral contraceptives appears to be associated with a statistically significant increase in the incidence of tromboembolic disease[1,2]. Because platelets occupy a central role in thrombus formation[3], many investigators have directed their attention towards the in vivo effects of oral contraceptive steroids on various aspects of platelet behavior[3] (e. g. platelet aggregation, adhesion and electrophoretic mobility). For example, ADP-induced aggregation of blood platelets is increased after 1 week treatment with estrogens alone[4] but only after treatment for 4 or more menstrual cycles with combination oral contraceptives[5,6]. Although investigators have reported that progestins produced no change in platelet aggregation[6,7], a recent long term study showed that after 2 years treatment with chlormadinone acetate aggregation was significantly accelerated[8].

The present study is the first report on the in vitro effect of steroids with estrogenic and progestonic activity on platelet aggregation.

Experimental methods. Blood was collected from female Syrian Random strain guinea-pigs and the platelet rich plasma (PRP) was obtained as previously described[9,10]. PRP was diluted to the desired platelet number with modified Tyrodes solution (Ca^{++}, Mg^{++}-free) pH 7.4 [composition per litre: 9.0 g NaCl, 0.2 g KCl, 1.0 g dextrose, 0.05 g NaH_2PO_4 and 1.45 g *Tris*[11]].

All test compounds were prepared as 30 mM aqueous dispersions by homogenizing in modified Tyrodes solution and stored at 0–5 °C.

Platelet aggregation was followed at 37 °C by employing a modification of the turbidometric method of BORN[12] as previously described[9,10,13,14]. Both 'sample' and 'reference' cuvettes in the Beckman DBG spectrophotometer contained PRP (0.4–0.6 ml) diluted with modified Tyrodes to a total volume of 2.25 ml and the recorder was arbitrarily set at 100% transmission. The test compounds were preincubated for 10 min by adding 0.25 ml of the aqueous dispersion to the 'sample' cuvette and 0.25 ml of modified Tyrodes to the 'reference' cuvette to compensate for volume changes. The final volume in each cuvette was 2.5 ml with a final platelet concentration of 150,000 platelets/cu/mm. Addition of the test compound (final concentration of $3 \times 10^{-3} M$) caused an instantaneous drop in the % transmission. At the end of the preincubation period 0.25 ml of $1 \times 10^{-5} M$ ADP (final concentration of $9.1 \times 10^{-7} M$) was added to the 'sample' cuvette and 0.25 ml of modified Tyrodes to the 'reference' cuvette and changes in light transmission were monitored. Appropriate controls were obtained be measuring the degree of platelet aggregation caused by a similar concentration of ADP in the absence of any added test compound. Aggregation is defined as the change in the % transmission after the initial instantaneous drop in light transmission due to platelet swelling or from the time of ADP addition when swelling did not occur.

Results and discussion. Because all the drugs used in this study were virtually water insoluble and because most of the organic solvents in which these compounds are soluble have an effect on platelet swelling and aggregation induced by ADP, aqueous dispersions of the various drugs were used. The use of dispersions in the present study is not unique. For example the effect of insoluble agents

Effect of several drugs on ADP-induced platelet aggregation

Drug	Effect of ADP-induced aggregation
Norethindrone	+[a]
Medroxyprogesterone Acetate	+
Estrone	+
Ethinyl Estradiol	+
Mestranol	+
Diethyl Stilbestrol	−[b]
Hexestrol	−

[a] Stimulation of aggregation.
[b] Inhibition of aggregation. Incubation conditions and concentrations as described in experimental methods.

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