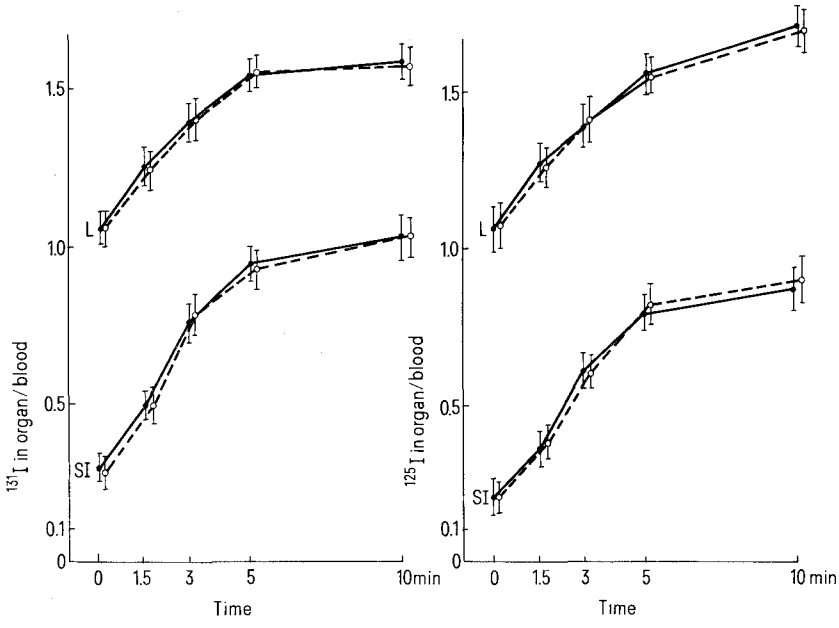


Fig. 2. Organ per blood ratios in the lung (L) and the small intestine (SI) of saline (●—●) or heparin (○—○) treated rats at various intervals after the challenge of anaphylactic shock. Each point represents the mean ± SE of 3 experiments.

well as in other organs (not included in Figure 2) of heparinized and non-heparinized rats. These findings mean that intravascular clotting as an etiological factor in the anaphylactic shock of the rat can be excluded.

A high dose of ellagic acid, a potent activator of Hageman factor, was necessary for even a transient fibrin formation (Figure 1); the distribution of the fibrin formed in the 1.5th min: liver 72%, lung 18%, small intestine 10%, which was readily eliminated later probably by the activated fibrinolytic system[3]. The anaphylactic results presented above show that the anaphylactic activation of Hageman factor, which we have demonstrated earlier in the rat[3], does not result in fibrin formation, although several additional etiological factors of hypercoagulability, such as anoxia and anoxaemia, endothelial damage, proteolytic enzymes and activated complement[12] are also existing. Consequently, highly potent anticoagulant activities are present or emerged even in the very early phase of anaphylactic shock, preventing intravascular clotting, and on the base of our present knowledge, it may be concluded that the slightly decreased fibrinogen level[4,5], the appearance of fibrinogen degradation products[6] and the marked hypocoagolability in the later phase of anaphylactic shock[3,4] are the consequences of the primary activation of fibrinolytic system[3,5].

[12] D. C. McKay, in *Coagulation* (Eds. S. Gottfried and P. Strandjord, 1973), p. 45.

Comparison of a Natural Heparinoid with Sodium and Calcium Heparin for their Effect on the Inhibitor of Activated Factor X

P. M. Mannucci, C. di Santo and F. Franchi

Hemophilia and Thrombosis Centre Angelo Bianchi Bonomi, Ospedale Policlinico, Via Pace 15, I-20122 Milano (Italy), 26 April 1976.

Summary. The reaction between activated factor X (Xa) and its natural inhibitor (XaI) was accelerated in vitro by both sodium heparin and an heparinoid, which was about 3 times less potent than heparin. The s. c. administration in humans of 5,000 units of sodium and calcium heparin was followed by the detection of a plasma activity potentiating XaI. In the majority of subjects, the heparinoid was not effective. These observations indicate that the use of heparinoids should not be considered as an alternative to heparin in the prevention of thromboembolism.

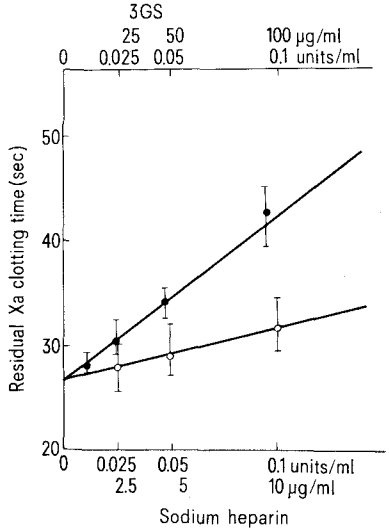
In the last few years heparinoids have received increasing attention for their potential applicability in the prevention and management of thrombosis and atherosclerosis. These compounds are acid mucopolysaccharides obtained by extraction from animal and vegetal sources or by semisynthetic procedures. Investigations largely carried out in animals have shown that they protect against experimental hyperlipidemia[1], release clearing factor into the blood stream[2], enhance fibrinolysis[3–5] and inhibit platelet aggregation[6–8], whereas blood coagulation screening tests[2,9] are hardly affected. However, small doses of heparin (0.2–0.01 U/ml) which are without effect on such coagulation tests, potentiate the natural inhibitor of activated factor X[10–12]. Since this is presently considered a key factor in the development of thrombosis[13], it would be of interest to know whether heparinoids mimick the effect of minidose heparin and enhance the plasma inhibitor activity. In this study, we have compared sodium and calcium heparin with a natural heparinoid extracted from pig duodenum, both in vitro and following their s.c. administration in human volunteers.

Materials and methods. The investigation was carried out in 6 healthy persons (aged from 24 to 33 years) who gave informed consent. The drugs tested were calcium

heparin, supplied in ampoules containing 25,000 units/ml; sodium heparin, supplied in ampoules containing 5,000 units/ml corresponding to 50 mg/ml; and glucuronyl-glucoseamin-glycon sulphate (3GS), supplied in vials containing 60 lipase units/ml of active substance corresponding to 6 mg/ml. The 3 drugs were administered s.c., in a random order, to the 6 volunteers with a free interval of 1 week between each treatment. Venous blood was collected in 3.8% Na citrate (9 parts of blood to 1 part of anticoagulant) immediately before the administration and then after 1, 3, 5 and 7 h. Plasma was harvested after centrifugation at 2,500 × *g* for 20 min at 4 °C, samples stored at –70 °C and tested within 2 weeks. Preliminary studies showed no difference between values obtained with plasma assayed immediately and after storing.

Measurement of plasma heparin (U/ml) in healthy volunteer subjects

Sample time (h)	Drug administered: Sodium heparin (mean/range)	Calcium heparin (mean/range)	3GS (mean/range)
Preheparin	0	0	0
Postheparin			
1	0.07 (0.04–0.09)	0.05 (0.03–0.07)	0.003 0–0.03
3	0.08 (0.03–0.09)	0.10 (0.03–0.15)	0.01 0–0.02
5	0.06 (0.03–0.12)	0.09 (0.03–0.15)	0.004 0–0.01
7	0.02 (0.01–0.12)	0.02 (0.01–0.03)	0.005 0–0.01
9	0 (0–0.01)	0	0



Effect of sodium heparin (○) and 3GS (●) on Xa inactivation in the presence of excess Xa inhibitor. The following reactants were added in order to a test tube at 37 °C: 0.5 ml of test sample (prepared by diluting with normal PPP heparin and 3GS solutions of known concentrations), 0.1 ml of normal PPP (as a source of XaI), 0.3 ml of *Tris* maleate buffer 0.02 *M* pH 7.5, and 0.1 ml of stabilized bovine Xa. At approximatively 90 sec after addition of Xa, 0.1 ml of the mixture was added to a second test tube at 37 °C followed by 0.1 ml of $CaCl_2$ 0.025 *M*; exactly at the end of the 120 sec incubation period, 0.2 ml of a mixture in equal volumes of human brain cephalin and anticoagulant-free bovine plasma were added and the residual Xa clotting time was then recorded. Each point of the curve represents the mean clotting time; the bars show the range observed in 4 experiments.

The heparin assay, which was carried out using a commercial kit (Sigma Technical Bulletin No. 870) exactly as described by the manufacturers, is based on the accelerating effect of trace amounts of heparin (or heparin-like substances) on the neutralization of activated factor X (Xa) by the plasma inhibitor to Xa (XaI) [13]. The assay system consists of a primary reaction mixture in which the test plasma is incubated for 2 min with Xa in the presence of excess XaI; the residual Xa is then measured in a secondary reaction mixture and the quantity of neutralized Xa converted to heparin concentrations (in U/ml of plasma) by comparison with a reference dilution curve prepared with known concentrations of sodium heparin. The sensitivity of the method is 0.01 U/ml.

Results and discussion. The Figure shows the in vitro effect on the heparin assay of various concentrations of 3GS compared with sodium heparin examined in the same experimental conditions. The reaction between Xa and its plasma inhibitor was accelerated by trace amounts of sodium heparin; 3GS was also effective, and 0.03 lipase units gave the same prolongation of the clotting time as 0.1 unit of heparin. However, since 1 unit of 3GS corresponds to 100µ g of active substance and 1 unit of heparin to 10 µg, 3GS appears to be about 3 times less potent on a weight basis.

The s.c. administration in humans of 5,000 units (50 mg) of sodium and calcium heparin was consistently followed by the detection of a plasma activity potentiating XaI (Table). The peak activity was observed between 1 and 3 h after injection, and measurable amounts were detectable until the 7th; these results are similar to those reported by Yin, Wessler and Butler[13]. There was no significant difference between sodium and calcium heparin both with respect to the levels achieved in plasma and their duration. In the majority of cases 3GS administered at the manufacturer-suggested dosage for therapeutic use (180 lipase units, 18 mg) was not followed by the appearance of a plasma activity potentiating the inhibitor; however, a weak activity (0.01–0.03 U/ml), close to the limit of sensitivity of the method, was occasionally shown in a small number of samples.

[1] P. Bianchini, V. Mantovani, E. Lo Marco and L. Bufalino, Arch. Pharmak. *269*, 470 (1971).
[2] P. Bianchini, G. Guidi and B. Osima, Biochem. exp. Biol. *10*, 243 (1972).
[3] I. Konttinen, Scand. J. clin. Lab. Invest. *14*, 87 (1962).
[4] A. Gibelli, C. Parotti, E. Bolandrina, F. Nicrosini and P. de Nicola, Farmaco *21*, 596 (1966).
[5] G. Prino and M. Mantovani, Eur. J. Pharmac. *6*, 190 (1969).
[6] J. Aznar, M. Lasso and A. Fernandez, Nature, Lond. *213*, 1251 (1967).
[7] J. F. Mustard and M. A. Packham, Pharmac. Rev. *22*, 97 (1970).
[8] M. Mantovani, Arzneimittelforsch. (Drug Res.) *23*, 239 (1973).
[9] C. C. Iliescu, L. Roma, V. Pastiu and I. Michel, Cor Vasa *4*, 225 (1962).
[10] E. T. Yin and S. Wessler, Biochim. biophys. Acta *201*, 387 (1970).
[11] E. T. Yin, S. Wessler and P. J. Stoll, J. biol. Chem. *246*, 3703 (1971).
[12] S. Wessler and E. T. Yin, Circulation *47*, 671 (1973).
[13] E. T. Yin, S. Wessler and J. V. Butler, J. Lab. clin. Med. *81*, 298 (1973).

At the dosage of 5,000 units given every 8 h after major surgery, heparin is known to prevent deep vein thrombosis and pulmonary embolism[14]. The potentiating effect on Xa inhibitor shown by the heparinoid in the in vitro system was lower than that displayed by sodium and calcium heparin. Hence, the absence of a measurable effect in plasma after its administration in humans at the highest dosage suggested for therapeutic use by the manufactures (18 mg) is not surprizing. On the basis of our in vitro studies, the administered dose should be 8 times higher in order to achieve the same effect of 5,000 units of heparin. These findings indicate that the use of heparinoids can hardly be considered as an alternative to heparin in the prevention and treatment of thromboembolism.

[14] V. V. KAKKAR, T. P. CORRIGAN and D. P. FOSSARD, Lancet *2*, 45 (1975).

Effects of Stress and Adrenocorticotrophin Administration on Plasma Corticosterone Levels at Different Stages of Pregnancy in the Mouse

SUSAN M. BARLOW, A. A. QUYYUMI, D. V. RAJARATNAM and F. M. SULLIVAN

Department of Pharmacology, Guy's Hospital Medical School, London, SE1 9RT (England), 5 April 1976.

Summary. Stress or administration of ACTH to pregnant mice gave rise to much higher plasma corticosterone levels in the second half of pregnancy than in the first half, suggesting that there may be increased adrenal sensitivity to ACTH or decreased metabolism of corticosterone during the second half of pregnancy.

In the mouse, resting plasma corticosterone levels increase markedly during the second half of pregnancy, reaching levels of around 140 μg/100 ml by day 16, that is 60 times the resting level found in the non-pregnant mouse[1]. Acute or chronic stress during this period of pregnancy results in further large increases, individual plasma corticosterone levels ranging from 500 to 900 μg/100 ml 1 h after the start of stress[2]. These large increases in plasma corticosterone levels could be due to the release of more adrenocorticotrophin (ACTH) in response to stress, or an increase in the sensitivity of the adrenal glands to circulating ACTH, or a reduction in the rate of metabolism of corticosterone, during the second half of pregnancy. It is not possible to measure adrenal corticosterone secretion rates accurately in an animal as small as the mouse and so an indirect approach was adopted to investigate the possible cause(s) of the high plasma corticosterone levels following stress during the second half of pregnancy, and to ascertain on which day around mid-pregnancy the corticosterone response to stress begins to increase.

In the first series of experiments mice were subjected to restraint stress by immobilizing them according to the method of RENAUD[3], on one of days 8, 10, 12, 14 or 16 of pregnancy (day of finding of vaginal plug designated day 1). Non-pregnant controls were similarly restrained. After 1 h of restraint stress blood samples were taken from the retro-orbital sinus under brief ether anaesthesia

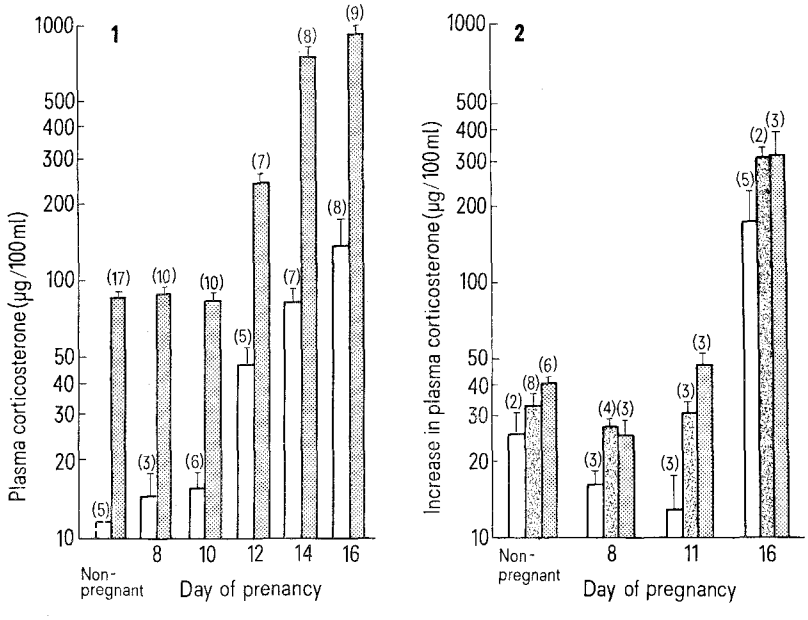


Fig. 1. Plasma corticosterone levels in control (white columns) and stressed (black columns) mice during different stages of pregnancy. The number of mice studied is shown in parentheses. Means ± SEM. The non-pregnant control level was 2.3 μg/ml.

Fig. 2. Increase in plasma corticosterone levels following injection of 40 mIU (white columns), 160 mIU (stippled columns), or 640 mIU (black columns) of ACTH during different stages of pregnancy in mice with endogenous corticosterone levels suppressed to 7–10 μg/100 ml plasma (except day 16, 43 μg/100 ml plasma). The number of mice studied is shown in parentheses. Means ± SEM.