Mucolytic Enzyme Systems XIX. Comparison of Hyaluronidase Inhibitor and Heparin Levels in Serum.* (19877)

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Because evidence was obtained suggesting that heparin might be a component of the non-specific hyaluronidase inhibitor in blood serum(1), determination of the degree of parallelism between the serum concentrations of heparin and inhibitor was investigated. For this purpose rabbits were subjected to two experimental procedures which had been reported to change the heparin or antithrombin concentration of the blood, and measurements of heparin and inhibitor levels as a function of time were carried out. The first procedure was suggested by the finding of Volkert(2) that the elevation in antithrombin activity of blood resulting from peptone shock could be inhibited by blocking the reticuloendothelial system with India ink. Accordingly, the effect of peptone shock with and without India ink injections was studied. The second procedure was suggested by the report of Bell and Stuart (3) that desoxycorticosterone alleviated "hyperheparinemia" in a clinical case. The simultaneous effect of this hormone on the heparin and inhibitor concentrations in the blood was measured.

In addition to these two *in vivo* studies, the *in vitro* effects of certain phenazine, oxazine and thiazine dyes, and also of aureomycin, on the heparin and inhibitor content of blood serum were determined because Haley and Stolarsky(4) showed that the dyes in question counteracted the anticoagulant action of heparin *in vitro,* and Waisbren and Glick(5) showed that aureomycin caused a decrease in heparin concentration in human plasma *in vitro.* The influence of the dyes and aureomycin on testicular hyaluronidase was determined, incidentally.

Methods. The procedures used for obtaining the rabbit serum, storage in the deep-frozen state, and the viscosimetric assay for hyaluronidase and its non-specific inhibitor have been indicated previously(6). Details of the assay(7,8), as well as of the mode of expressing the percent inhibition(9), may be found in earlier papers of this series. The rabbits served as their own controls in the *in vivo* experiments since comparisons were made on the same animals before and after the treatments. For each analysis of the serum inhibitor, 0.025 cc of serum was used; however in the dye and aureomycin experiments 0.040 cc of serum was employed to magnify the effect of these agents on the inhibitor. The dye solutions were adjusted to pH 6.7 with 0.2 N NaOH before addition of the serum, and the aureomycin glycinate used was neutralized with 0.1 N HCl to pH 7.3. *Heparin* was determined by the method described by Jaques, Monkhouse and Stewart (10), which was based on an earlier study of Jaques *et al.*(11). The principle of the method is spectrophotometric measurement of the metachromatic color produced by the reaction of heparin with azure A, after purification of the heparin by precipitations with octylamine and brucine. As used in this study, certain details have been modified to sharpen the analytical precision as follows: To each 5-10 cc sample of serum, 1 cc 7% octylamine hydrochloride (Sharples) was added, mixed, and after a few minutes centrifuged at 1700 times gravity and –2°C for 15 minutes. The decreased temperature and increased time and speed compared to the original method made for more complete precipitation of the heparin complex. (For some human sera, particularly those from patients with nephrosis, heating at 56°C for 30 minutes before addition of the octylamine resulted in more

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TABLE I. Effect of Peptone Shock on Hyaluronidase Inhibitor and Heparin Concentrations in Rabbit Serum.

Treatment and time of sampling	No. of rabbits	Mean % inhibition	Mean μg % heparin
Before peptone inj. (1 g/kg) i.v.	7	24 ± .5	230 ± 8
.5 hr after peptone inj.	7	35 ± 1.4	480 ± 15
24 " " " "	6	32 ± 1.6	220 ± 16
72 " " " "	5	28 ± 2	230 ± 19
Before India ink inj. (.5 cc/kg) i.v. and 3 wk after end of above exp.	6	20 ± 1.1	220 ± 9
.5 hr after India ink inj.	6	19 ± 2.6	210 ± 37
24 " " " " and just before peptone inj.	6	17 ± 1.1	210 ± 10
.5 hr after peptone inj. (1 g/kg)	6	11 ± 1.6	130 ± 15
24 " " " "	6	18 ± .8	210 ± 10
72 " " " "	6	22 ± .3	200 ± 12

TABLE II. Effect of Intramuscular Injection of Desoxycorticosterone Acetate on Hyaluronidase Inhibitor and Heparin Concentrations in Rabbit Serum.

Treatment and time of sampling	No. of rabbits	Mean % inhibition	Mean μg % heparin
Before 1st inj. (25 mg DOCA in 5 cc sesame oil per day for 4 days)	10	16 ± .4	220 ± 2
Before 4th inj.	10	19 ± 1.2	310 ± 29
.5 hr after 4th inj.	10	25 ± 2.5	350 ± 34
2 " " " "	9	22 ± 1.4	290 ± 42
72 " " " "	10	23 ± 1.4	280 ± 29
120 " " " "	9	18 ± 1.3	250 ± 27
Before 1st inj. (5 cc sesame oil/day for 4 days) and 3 wk after end of above exp.	6	16 ± 0	250 ± 27
Before 4th inj.	6	18 ± 1	280 ± 20
.5 hr after 4th inj.	6	17 ± .5	280 ± 7
2 " " " "	4	17 ± .9	280 ± 0
24 " " " "	5	19 ± 1.3	300 ± 20
72 " " " "	6	17 ± .8	220 ± 39

complete precipitation. This was not true for the rabbit sera.) The treatment of the heparin complex followed that given in the original procedure(10) to the point of addition of phosphate buffer to the alkaline hydrolysate of the heparin-brucine precipitate. At this point 1 cc of 0.15 M KH_2PO_4 and 1 cc of water were added to the 2 cc of heparin in 0.05 N NaOH, the solutions were centrifuged for 10 minutes at about 15,000 times gravity to remove cloudiness that was occasionally encountered, 1 cc of azure A solution was introduced and the pH was checked with a glass electrode. If the pH was not 7.3 it was adjusted to this value. Usually not more than a drop of 0.05 N NaOH was sufficient for this purpose. Somewhat greater color development occurred at this pH than in more alkaline solutions. The azure A (National Aniline, 92% dye content) employed was made up to a 100 mg% stock solution every 2 months and it was diluted 1:9 before use. After addition of the azure A the optical density was measured at 530 mμ with a Coleman spectrophotometer. Blank and standard determinations were run in which physiological saline solution or heparin (Hynson, Westcott, and Dunning) solutions of known concentration were substituted for the serum. A linear relationship between optical density and amount of heparin was found over the range 10-60 μg, and the serum samples were adjusted to yield an amount of heparin in this range. Satisfactory recoveries of heparin added to serum were obtained.

After the present studies were almost completed, a report appeared by Monkhouse and Jaques(12) in which they applied to plasma the method of Homan and Lens(13) of separating heparin from protein by phenol, and a

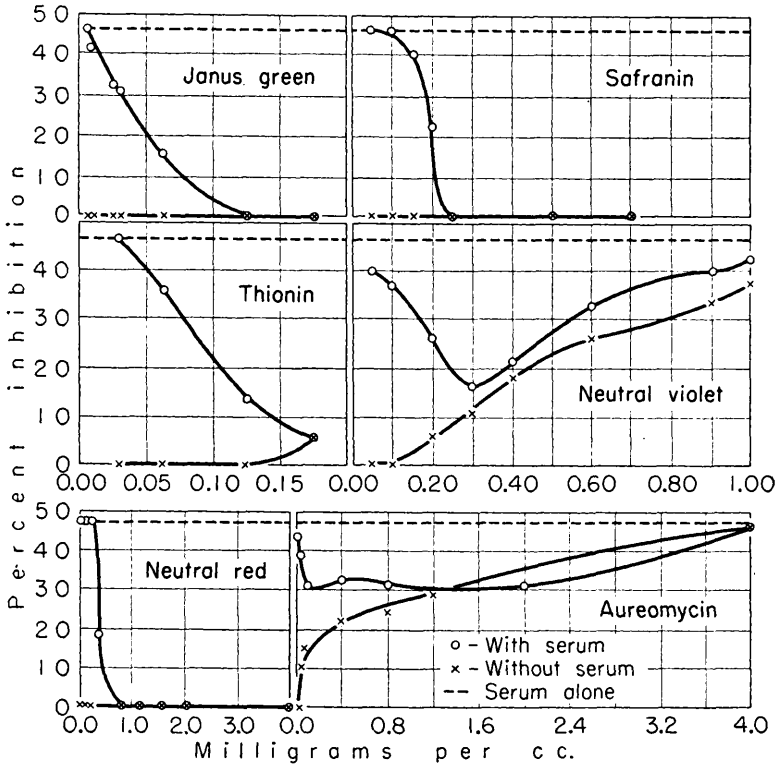

FIG. 1. Effect of dyes and aureomycin on the activity of non-specific hyaluronidase inhibitor of serum and testicular hyaluronidase.

further modification of the method was described later by Gibson *et al.*(14) who employed zinc hydroxide for the reprecipitation of the heparin.

Peptone shock experiments were carried out by administering single doses of 10% peptone (Merck) in 0.9% NaCl to the rabbits via ear vein and withdrawing blood at intervals as indicated in Table I. The peptone solution was freshly prepared, boiled for 10 minutes, cooled, and adjusted to pH 7.4 before injection by sterile technic. In a second group of experiments India ink (Higgins) was administered to the same animals after a period of recovery, subsequently they were given another peptone injection, and blood samples were taken at the intervals indicated in Table I. Administrations of desoxycorticosterone acetate in sesame oil ("Percorten", Ciba), and control injections into some of the same animals with oil alone, were conducted as indicated in Table II; the blood samples were withdrawn for analysis at the intervals given. In the *in vitro* dye and aureomycin experiments the ranges of concentration of these substances that were employed are given in Fig. 1. In all cases the solutions were added to the serum before other components of the reaction mixture.

Results. From the data in Table I it is apparent that the peptone shock elicited a significant maximum elevation of the serum concentrations of both the inhibitor and heparin within 0.5 hour. The heparin concentrations fell to the preinjection level after 24 hours but the inhibitor level dropped more slowly and was still slightly elevated after 72 hours. India ink injection had no perceptible effect on the heparin concentration but caused a slight fall in the inhibitor, and subsequent peptone shock produced a decrease in both of these factors within 0.5 hour (instead of the rise observed from the same degree of shock without previous ink-block) with a return to the preshock level within 24 hours.

A degree of parallelism was found in the rise of the inhibitor and heparin concentrations following desoxycorticosterone injec-

tions, Table II. A maximum was found, 0.5 hour after the fourth injection, while practically no effect was observed in the control experiment with sesame oil alone.

The data in Fig. 1 show that janus green, safranin, thionin, and neutral red have no direct effect on hyaluronidase activity under the conditions employed. Inhibition of the enzyme was obtained with neutral violet and aureomycin. However, all of the dyes reduced the effect of the serum inhibitor. In the case of neutral violet and aureomycin this latter effect was counteracted and, at higher concentrations, overcome by direct inhibition of the enzyme. With respect to concentration required to inhibit the serum inhibitor, janus green and thionin were more effective than safranin, neutral violet or neutral red.

Discussion. The present data indicate a general parallelism in the direction of the changes that have been made to occur in the heparin and inhibitor, but correlation of magnitude and time of change was somewhat variable. This might be due, at least in some degree, to the nature of the heparin analysis which can include other acid polysaccharides in addition to the heparin. The rise in heparin concentration that was observed following desoxycorticosterone injections contrasts with the finding of Bell and Stuart(3) that the hormone reduced the level of heparin or heparinoid substances in the blood of a patient with "hyperheparinemia" as measured by clotting tests and protamine titration. On the other hand clinical evidence, such as that given and cited by Margulis(15) and Smith *et al.*(16), that particular hormonal or stress stimulations of the adrenal result in a decrease in clotability of blood in some cases may be related to the present data as well as to the elevation in the level of the inhibitor in serum that these adrenal stimuli also produce(6).

The data obtained by *in vitro* experiments with the basic dyes, along with data previously obtained with toluidene blue(8), show that the dyes most effective in reducing the activity of the serum inhibitor are those which Haley and Stolarsky(4) found most effective in reducing the anticoagulant action of heparin in rabbit plasma. Thus, Janus green, thionin, and toluidene blue, which had similar effects on the inhibitor, were more effective for a given dye concentration than safranin, neutral violet and neutral red.

The earlier data of Waisbren and Glick(5) on the role of aureomycin in decreasing heparin concentration *in vitro*, and lowering coagulation time when administered *in vivo*, is supplemented by the present data showing that the antibiotic also reduces the serum inhibitor *in vitro*.

Summary. The effects of peptone shock (with and without block of the reticuloendothelial system by India ink), and administrations of desoxycorticosterone on the nonspecific hyaluronidase inhibitor and heparin concentrations in the blood serum of rabbits at intervals before and after the treatments were observed. A general parallelism was noted in the direction of the changes produced in both serum factors, but variations were observed with respect to the magnitude and time of change. *In vitro* experiments were carried out on the effect of janus green, thionin, toluidene blue, safranin, neutral violet, neutral red, and aureomycin on the serum inhibitor and on hyaluronidase. A general correlation was observed between the anti-heparin action of these substances and their anti-inhibitor effects.

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Motile Life of Bovine Spermatozoa in Glycine and Yolk-Citrate Diluents at High and Low Temperatures. (19878)

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In experiments with sea urchins and other invertebrates it has been found(1,2) that the addition of any one of a number of amino acids or peptides to the spermatozoa, diluted in sea water, extends very greatly their duration of motility and fertilizable life-span. The extension of functional life-span is accomplished(3) without metabolic utilization of the added amino acid. Experiments with fowl sperm(4) have also shown an extension of motile life-span as a result of the addition of an amino acid to the saline diluent. It was of interest, then, to learn whether or not such agents would be effective in extending the life-span of mammalian spermatozoa. The experiments with the invertebrates were performed at temperatures in the range of their usual environment. Life-span studies on spermatozoa of mammals have generally been carried out at low temperatures since the purpose evidently is to extend, as much as possible, the period during which the sperm may be stored before use in artificial insemination. It should be noted, however, that mammalian spermatozoa must survive at body temperature in the female genital tract for some time after insemination before the eggs are encountered. Their duration of survival at such temperature may, then, be an important factor in determining whether or not successful fertilization will occur.

In view of these considerations the present experiments with bovine spermatozoa were performed at both body and refrigerator temperatures. The effect of the amino acid solution was compared with that of isotonic NaCl, balanced salt solution and the yolk-citrate diluent(5) which, following the discovery(6) of the efficacy of hen's egg-yolk, is generally employed for storage of bovine spermatozoa at low temperatures.

Material and methods. Semen was obtained from Holstein and Guernsey stud bulls of the Roger Jessup Certified Farm† in Los Angeles, Calif. The samples were gradually cooled and kept at 2° to 4° until use at the laboratory, usually between 1¾ and 6¼ hours later. Sperm counts of the various samples ranged from 1 to 14 x 10^8 per ml. The semen was diluted 1/100 and 1/1000 in the various test solutions. Pyrex, glass-stoppered, 25 ml Erlenmeyer flasks were used with 4 ml of the diluted semen. In the experiments at 38.6°C, these were shaken at 80 c.p.m. and 5 cm excursion. In the experiments at 4°C, 50 ml flasks were used with 4 ml of diluted semen and these were not shaken. Estimates of the percentage of motile sperm were made hourly in the 38.6°C series and daily in the 4°C series, with 2 or 3 readings taken during the first period. Motility was scored as 0, 1, 5,

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