Photometric Assay of Plasma Heparin.* (19452)

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Chemical methods for the quantitative determination of heparin in blood plasma are based on the changes in certain basic dyes added to at least partially separated metachromatic substance. Heparin seemingly is not found in normal blood plasma, though traces of other mucoitin sulfates are present. It is possible to follow by heparin estimations the plasma level when heparin is administered and to recover heparin added to blood plasma. Gibson *et al.*(1) reported briefly a separation and photometric procedure for estimating heparin in blood plasma. Further details and some applications are now presented. The concentration of heparin is expressed as anticlotting units in terms of the weight of certain purified standards, *e.g.*, those of the International Provisional Standard and of the Connaught Laboratory. The U. S. Pharmacopoeia now defines a unit as approximately the quantity of heparin sodium required to maintain fluidity in 1 ml of plasma prepared according to the directions of the U.S.P. assay for heparin sodium. We are using for a standard an Upjohn heparin sodium assaying 130 units per mg. Metachromatic heparin assay was proposed by MacIntosh(2) who used purified preparations in concentrations of about 0.01% and a provisional British standard. He measured the loss of color of an aqueous toluidine blue O solution (with a Spekker absorptionometer) after separating the red precipitate by overlaying the mixture with petroleum ether. Jaques, Mitford and Ricker(3) assayed solutions of heparin prepared according to Charles and Scott(4), measuring the change in color of azure-A with a Lovibond tintometer. Jaques, Morehouse and Stewart(5) precipitated the "heparin-protein-complex" from citrated plasma with n-octylamine, reprecipitated the heparin with brucine phosphate, and quantitated it as above. Good recoveries of added heparin were obtained, but with small quantities and with normal plasma difficulties were encountered. Then Monkhouse and Jaques(6) adapted a phenol separation of heparin from plasma devised by Homan and Lens(7) for metachromatic assay. The metachromatic assay of purified heparin offers little difficulty. The addition of toluidine blue O or azure A to a heparin sodium solution (pH 7.4-7.8) results in a flocculation of a reddish purple precipitate. This can be centrifuged and the precipitate washed and extracted with alkaline alcohol. The resulting red solution may be read photometrically.

Method. A separation of the heparin from plasma or serum is essential. The following procedure when carefully followed is satisfactory. *Special reagents.* n-octylamine (Sharples) 2 ml, concentrated hydrochloric acid 1 ml, and water 30 ml, adjusted to a pH of 7.3-7.5; 0.5 N sodium hydroxide and exactly 8.05% (0.5N) zinc sulfate; toluidine blue O solution, 100 mg in 100 ml of water, diluted 4:10 and centrifuged before using.

Five ml of oxalated plasma in a 15 ml test tube are diluted with 5 ml of pH 6.1 M/15 phosphate buffer. Then 2 ml of n-octylamine hydrochloride solution are added in 0.5 ml

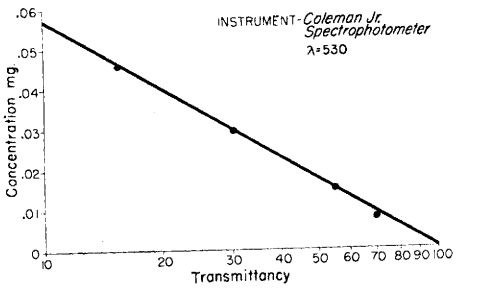

FIG. 1. Standard curve, Upjohn, sodium heparin (130 units per mg).

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portions at 5-minute intervals with immediate mixing. The tube is centrifuged as soon as flocking is observed. If flocking is delayed over 10 minutes, the tube may be warmed to 40°C. The tube is centrifuged, the supernatant poured off, and the precipitate washed by suspension in 10 ml of water and again centrifuged. The precipitate is moistened with exactly 0.5 ml of 0.5 N sodium hydroxide solution, mixed with 10 ml of 0.9% saline and heated at 60-70°C in a water bath for 15 minutes. When cool, 0.5 ml of 0.5 N zinc sulfate solution is added and mixed and the tube again centrifuged after 30 minutes. The heparin is contained in the precipitate. It is extracted with 5 ml of water plus 5 ml of 7.8 phosphate buffer and heated at 65-70°C with stirring for 15 minutes. The tube is centrifuged and the supernatant drained into a fresh test tube. To this is added 1 ml of the toluidine blue O solution. A reference blank with water-buffer mixture and dye is made. Both tubes are allowed to stand for several hours, or better overnight. A red precipitate forms in the heparin tube. The heparin tube is centrifuged, drained, and the red precipitate washed in 5 ml of water and again centrifuged. The reference tube is similarly washed. The inside walls of both tubes after draining are swabbed with alcohol-moistened and dry gauze sponges. Four ml of alcohol and 1 ml of 0.1 N sodium hydroxide are added to each tube. After 20-30 minutes the heparin tube is centrifuged and the supernatant read at 530 mμ in a Coleman Jr. spectrophotometer (75 x 12 mm cuvettes) with the dye blank tube set at 100% transmittancy.

A calibration curve for heparin sodium is prepared. The standard should contain 10 ml of heparin sodium (Upjohn) in 100 ml of saline 0.9%. Four portions: 0.01, 0.02, 0.03 and 0.04 mg of heparin sodium are added to 10 ml amounts of saline and precipitated with sodium hydroxide and zinc sulfate. The heparin is extracted and precipitated and the colored end solution obtained as above. The graph is a straight line (Fig. 1) when plotted on semi-log coordinates.

The metachromatic precipitate. Heparin enters into a combination with the dye to form the red-purple precipitate. If the red alkaline alcohol solution for the calibration curve is centrifuged, the trace of residue when taken up in phosphate buffer can be reprecipitated with the dye and again gives the red color of the same intensity with alkaline alcohol. This procedure may be repeated and further purification of heparin seems possible.

TABLE I. Recovery of Heparin Sodium Added to 5 ml of Plasma.

Plasma, mg	+.01 mg, mg	Recovered, mg
.011	.022	.011
.015	.024	.009
.003	.013	.010
.006	.019	.013
.027	.036	.009
.013	.025	.012
.008	.019	.011
.012*		.011*
	+.02 mg	
.006	.026	.020
.011	.029	.018
.006	.024	.018
.006	.025	.019
.002	.021	.019
.005	.026	.021
.005	.024	.019
.006*		.019*
	+.03 mg	
.008	.032	.024
.005	.036	.031
.003	.035	.032
.016	.045	.029
.010	.040	.030
.007	.037	.030
.007	.033	.026
.008*		.029*
	+.04 mg	
.013	.052	.039
.007	.050	.043
.005	.047	.042
.001	.039	.038
.010	.048	.038
.012	.055	.043
.012	.055	.043
.006*		.041*

* Average.

Recovery of heparin added to plasma. Determinations on 5 ml amounts of normal plasma to which had been added .010, .020, .030 and .040 mg of heparin sodium were done. Recoveries have been very satisfactory, averaging .011, .019, .029, and .041 mg for the 4 groups (Table I).

The normal plasma level of metachromatic substance as heparin sodium. Metachromatic

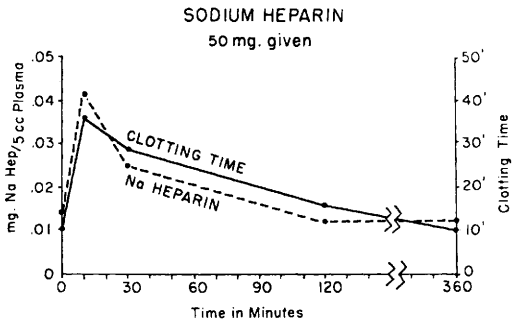

FIG. 2.

changes are induced by other mucoitin sulfates and esters (Lison)(8). Monkhouse and Jaques(9) are of the opinion that the metachromatic material in normal blood is not heparin. Significant amounts of metachromatic reacting substances are found in normal plasma, as may be seen from the plasma figures in Table I. These have amounted to .02 to .54 mg%. Phosphate saline extracts of 20 ml of pooled plasma were concentrated *in vacuo* and dialyzed against saline solution to remove the phosphates, and the anticoagulant activity tested by the Waugh-Ruddick(10) technic. There was no increase in the clotting time. If heparin was added to the plasma similarly treated, the heparin was recovered when assayed by effects on clotting time.

Plasma heparin levels and clotting times. The curve in Fig. 2 shows the plasma heparin sodium levels for 5 ml plasma samples and clotting times when heparin sodium was administered i.v., in a 50 mg dosage. The curve for the heparin contents parallels the clotting times. Depo-heparin 200 mg intramuscularly exhibits a prolonged but lesser clotting time increase and only a slight rise of the heparin level.

Urine. It has long been known that urine contains some chondroitin sulfuric acid in its non-dialysable constituents. Pons(11) has determined the daily output in the urine as 80 to 90 mg per day. If 1 ml of normal urine is diluted to 10 ml with water, precipitated with sodium hydroxide and zinc sulfate, the precipitate treated with pH 7.8 phosphate and water and toluidine blue O added to the supernatant, a considerable metachromasia is obtained. This amounts to 3 to 12 mg% expressed as heparin sodium. When equal parts of 3.5% saline instead of water and the pH 7.8 phosphate are used, there is no metachromatic effect with urine alone, but heparin added to the urine can be determined. For example, 1 ml of the urine treated as above gave by the water-buffer-dye precipitation .060 mg of metachromatic substance expressed as heparin sodium; with .025 mg of added heparin sodium, .084 mg was found. The 3.5% saline and buffer procedure was .00 mg for the urine, but .025 mg of added heparin sodium returned .026. A control with .025 mg heparin sodium assayed .026 mg. Astrup(12) used a benzidine precipitation of the heparin-like substance in urine (and plasma). This substance has a slight anticlotting effect, which he did not believe to be heparin. We have found little or no anticlotting effect in our metachromatic reacting material from normal urine.

Summary. A photometric assay of heparin sodium is described. This consists of a preliminary precipitation of 5 ml of plasma with n-octylamine, reprecipitation with zinc hydroxide, decomposition of this precipitate with phosphate buffer solution, and final precipitation with toluidine blue O. After washing, the red-purple residue is taken up in alkaline alcohol and read photometrically. The heparin is a component of the red-purple dye precipitate. Normal plasma contains non-active heparin-like substances, amounting to 0.02 to 0.54 mg%. Added heparin sodium to plasma is quantitatively recovered. Intravenous heparin sodium and "depo-heparin" levels follow the clotting inhibition effects. Normal urines contain from 3 to 12 mg% of non-active metachromatic substance expressed as heparin sodium. Heparin sodium added to urine may be recovered.

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Effects of Vitamin A Malnutriture on Resistance to Stress.* (19453)

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Considerable data are available indicating that resistance to stress may be significantly impaired in the nutritionally-deficient animal. Thus an impaired resistance to cold has been demonstrated in rats deficient in pyridoxine (1), riboflavin(2), and vit. A(3,4), and guinea pigs deficient in vit. C(5). An impaired resistance to anoxia has been reported in the riboflavin-deficient rat(6,7); and failure to excrete water and to withstand water intoxication has been observed both in riboflavin-deficient(8) and pyridoxine-deficient (9) rats. In the present communication data are presented on the effects of graded doses of vit. A on resistance to 1) low environmental temperature and 2) x-irradiation injury in the rat.

Procedure. The animals employed in the present experiment were male rats of the University of Southern California strain. The mother rats were maintained for at least 2 months previous to breeding and for 12 days after the birth of the litter on Sherman diet B(10) without addition of supplementary lettuce or meat. Litters were cut to 7 at 3 days of age, and on the 12th day mothers and litters were placed on a vit. A-low diet.† The young were weaned at 21-24 days of age and at a body weight of 36-50 g inclusive. The basal ration employed in the present experiment had the following composition: sucrose, 60%; casein,‡ 25%; salt mixture,§ 5%; cottonseed oil (Wesson), 10%; and the following synthetic vitamins per kg of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; ascorbic acid, 200 mg; biotin, 5 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 150 μg; 2-methyl-naphthoquinone, 10 mg; and choline chloride, 2 g. To each kg of diet were also added 400 U.S.P. units of vit. D.‖ Each rat also received once weekly a supplement of 4.5 mg alpha-tocopherol acetate. Animals were kept in metal cages with raised screen bottoms to minimize access to feces and were fed *ad libitum*. Diets were made up weekly and stored under refrigeration. Rats were fed on alternate days. All food not consumed 48 hours after feeding was discarded to minimize oxidative changes in the diet.

Results. Exp. 1. Effects of graded doses of vit. A on survival time of rats under cold room and room temperature conditions. Rats averaging 43.4 g in weight were divided into 5 groups of 30 each and were fed the basal vit. A-free ration. They received in addition,

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† Similar to U.S.P. XIII depletion diet except that commercial casein rather than extracted (vit. A-free) casein was employed.

‡ Vitamin Test Casein, General Biochemicals, Chagrin Falls, O.

§ Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

‖ HY-DEE Powder, Standard Brands, New York.