ponents common to both the immune system and the mechanism of blood coagulation.

Summary. 1. Heparin was found to inhibit *in vitro* the agglutination of red blood cells in 3 consecutive cases of idiopathic acquired hemolytic anemia. 2. Following incubation of patients cells with heparin, the auto-agglutination disappeared. The Coombs reaction also became negative while the anticoagulant activity of heparin greatly diminished. The heparin tolerance, tested in the first 2 cases, was abnormally high. 3. No interaction *in vitro* was observed between heparin and iso-agglutinins. 4. The possible mechanism, specificity and clinical value of the reaction is discussed, and certain implications with respect to the clotting mechanism are pointed out.

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Use of Anthrone Reagent to Estimate Inulin in the Presence of Glucose.* (21097)

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Young and Raisz(1) have demonstrated that Dreywood's anthrone reagent can be used for the quantitative determination of inulin. They concluded that the anthrone method had many advantages over other procedures currently employed. Studies in our laboratory during the past 2 years have led to a similar conclusion although difficulty was found in applying the anthrone procedure for the estimation of inulin in the presence of glucose. The resolution of this problem, important for biological experimentation involving inulin, is the main consideration of the present study.

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Direct anthrone technic for inulin. Morris (2) demonstrated that an intense heat needed to effect maximal color reaction was generated by the rapid addition of the anthrone reagent *directly* into an aqueous solution of most carbohydrates. With inulin, however, we found that the color formed with the anthrone reagent was maximal when we *suppressed* the heat of solution created by the direct intoduction of the anthrone reagent. This finding was applied to estimate microquantities of inulin in the following manner: Two ml quantities of standard aqueous solutions of inulin were introduced into Klett tubes which were immersed in a cool tap water bath for 10 minutes. Into the tubes 4 ml of 0.2% anthrone reagent were delivered rapidly by

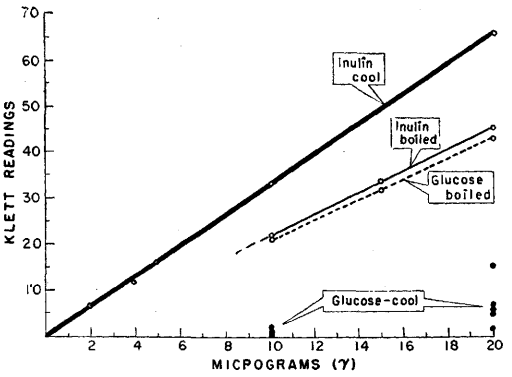

FIG. 1. Color formation by inulin and glucose with the "Direct Technic." Curve and dots labelled "cool" give Klett-Summerson readings ($\lambda = 620$ μ) when 4 ml of anthrone is blown rapidly into Klett tubes containing 2 ml aqueous solutions of inulin and glucose and cooled in tap water bath. Curves labelled "boiled" give Klett-Summerson readings after tubes were in boiling water bath for 10 min.

blowing the reagent out of 4 ml pipettes; the contents were thoroughly stirred with glass rods. After 2 or 3 minutes, the tubes were removed from the water bath, allowed to reach room temperature, and read in a Klett photoelectric colorimeter with a 620 mμ filter. The chief care required in the procedure was to obtain a consistent rate of delivery and thorough mixing of the anthrone reagent.

In Fig. 1 are shown *results* (labelled "cool") obtained with microconcentrations of inulin and glucose by this direct technic. Quantities of inulin from 2 to 20 μg led to color formation that was linear with the concentration. For a given concentration in this range, the optical density never varied more than 2% from one experiment to another. With glucose, however, the results were found to be totally inconsistent and unsatisfactory with this technic (Fig. 1, glucose-cool); the resultant optical densities varied with imperceptible and uncontrollable changes in the delivery of the anthrone reagent. Boiling for 10 minutes in a water bath resulted in a straight line curve for several concentrations of glucose, but it also lowered the sensitivity of the anthrone reaction for inulin as compared with the direct technic. This decreased sensitivity ruled out the boiling procedure except for large quantities.

Effects of alkali on inulin. The extreme simplicity and sensitivity of the direct technic for the estimation of small quantities of inulin made it worthwhile to explore methods to eliminate any accompanying glucose. Such a step was necessitated by the inconstant reactivity of glucose thus precluding the use of a "chromogenic blank" for correction even when a constant amount of glucose was present. Following the suggestion of Little(3), sodium hydroxide was employed to destroy glucose in the presence of inulin. This was attempted by adding 0.5 ml of 5 N NaOH to one ml quantities of aqueous standard solutions of inulin and glucose in Klett tubes. The tubes were heated in a boiling water bath for exactly 5 minutes, cooled, and neutralized with 0.5 ml of 5 N H_2SO_4. The anthrone reagent (4 ml) was then added as described in the "direct technic". Glucose was found totally destroyed by this procedure but solutions of inulin also showed some degradation. Solutions of inulin from 3 different sources which had similar optical densities for a given concentration when estimated by the direct technic showed varying diminution of optical densities (33%, 27%, and 17% respectively) when determined by the same technic after having been boiled in alkali. At the time we were unfamiliar with the work of Ross and Mokotoff(4) and Weil(5) who had demonstrated that commercial inulin preparations contained variable portions that were destroyed by alkali. Thus, further attempts were made to standardize the procedure by varying the strength of the alkali from 1 N to 10 N and by varying the boiling period from 2 to 10 minutes. The percentage of degradation after boiling in alkali seemed relatively constant for a single inulin source when treated under controlled conditions even though there were great variations among samples from various sources. Biological testing seemed indicated, and accordingly intravenous infusions of inulin were given to 2 patients. The urine and plasma filtrates† from

† Plasma filtrates prepared by addition of 0.2 ml of plasma to 2.8 ml dilute tungstic acid solution(6) in centrifuge tubes. Tubes were corked, vigorously shaken several minutes, and then centrifuged. One ml of supernatant was diluted with 1 ml of water for analysis.

these patients were analyzed for inulin by the direct anthrone technic after boiling in 5 N NaOH for 5 minutes. It was found that only 0 to 3% of the inulin in the plasma were degraded whereas 9 to 12% of the inulin in the urine behaved in similar fashion. In pursuing this problem further, samples of plasma and urine were obtained from patients undergoing inulin clearance studies in another hospital‡ and the inulin concentrations as determined in its laboratory by the Schreiner (7) method were ascertained. The analytical data obtained using the direct anthrone technic following the treatment of the urines and plasma filtrates with NaOH were calculated relative to an inulin standard which was *not* treated with alkali. The results again demonstrated the above-mentioned finding that the inulin in the plasma seemed more resistant to alkali than the inulin in the urine. There was an average of 2.4% degradation in the former compared to 15% in urine. Plasma filtrates prepared with N/200 acetic acid gave the same results, thus ruling out the tungstic acid used in precipitation as the cause. There was poor agreement of our results relative to those obtained by the Schreiner method and this was definitely due to the use of the alkali. The inconstant distribution of the fraction of inulin which is destroyed by boiling in NaOH precludes the use of this step to determine inulin during biological studies. Specimens of blood and urine containing equal quantities of inulin but with variable percentages of labile inulin would yield erroneous total values by the use of such an analytic procedure. Young and Raisz(1) have solved this problem by preparing for biological experiments an inulin which has been made stable in alkali by pre-treatment with sodium hydroxide. This allows the subsequent use of the anthrone method for the estimation of inulin following the destruction of glucose with sodium hydroxide. An alternate anthrone method has been developed in this laboratory which entirely eliminates the need to boil in alkali and has proven to be extremely accurate.

‡ We are indebted to Dr. B. Crawford of the N.Y.U.-Bellevue Medical College for plasma and urine specimens.

Indirect anthrone technic for inulin. This technic, proposed by Seifter *et al.*(8) for the estimation of glucose and glycogen, consists of two stages: first, the suppression of the heat generated by the addition of the anthrone reagent, and next the application of external heat to produce the color reaction under controlled conditions. Experiments in this laboratory revealed that when meticulous care was taken in the first step to eliminate *all* heat during the addition of the anthrone reagent, glucose in concentrations up to 250 μg gave *no* color. This overcame the difficulty encountered with the "direct technic" where glucose gave rise to variable intensities in color during the initial contact with the anthrone reagent. Three steps were found necessary for effective suppression and instantaneous absorption of the heat when mixing the anthrone reagent with an aqueous solution; practical application was not difficult. Two ml quantities of standard aqueous solutions were introduced into matched Klett tubes which were immersed in an ice water bath. A beaker with 95% sulphuric acid and one with anthrone reagent were also chilled. After 15 minutes, 2 ml of the chilled 95% sulphuric acid were allowed to flow slowly by gravity along the inside of each Klett tube from 2 ml delivery pipettes chosen for their slow outflow rates. This preliminary dilution with sulfuric acid was necessary because icing and slow introduction was not sufficient to suppress "flash" heat production that occurred when the 95% sulfuric acid in the anthrone reagent was pipetted into a completely aqueous solution. After thorough mixing with flat bottomed stirring rods, 2 ml of chilled 0.4%§ anthrone reagent was introduced into each tube in the manner previously employed when adding the 95% sulfuric acid. The contents of the tubes were again thoroughly stirred and the tubes were removed from the ice bath and allowed to come to room temperature. At this stage the glucose solutions had the same color as the water blank similarly treated with anthrone. In fact, this criteria served as a check as to

§ The anthrone reagent is double the strength usually used.

TABLE I. Development of Color by Inulin and Glucose Solutions at Varying Temperatures following Mixing with Anthrone Reagent by a Technic Which Had Suppressed Color Formation before Heating.

Heat and temp.		Time, min.	Klett-Summerson readings (at λ 620) Glucose 100 γ	Glucose 200 γ	Inulin 10 γ	Inulin 20 γ
Room temp.		30	.25	.0	13.3	24.5
Incubator	37°C	60	.0	.1	19.0	37.5
Water bath	40	30	1.3	2.5	32.0	61.0
Idem	50	20	5.0	10.0	37.5	74.0
"	56	10	4.5	9.1	37.2	74.5

Results of Analyses of Combinations of Glucose and Inulin (Color Developed after 10 Min. in Water Bath at 56°C). These are compared with the sum of results obtained separately for each carbohydrate.

γ glucose	γ inulin	Klett-Summerson readings Determined value	Calculated value
100	10	43.0	41.7
100	20	79.8	79.0
200	10	46.5	46.3
200	20	83.0	83.6

whether the proper technic was used. A slight color was usually noted at this stage with inulin samples but did not influence later results.

Subsequent heating gave reproducible and consistent color formation with both inulin and glucose solutions. Representative data obtained at various levels of heating are given in Table I. Young and Raisz(1) found that heating at 75°C for 5 minutes led to maximal development of color on the part of inulin. Since the aim of the present method was to suppress formation of color by glucose, lower temperatures were selected. At 56°C the color formation with inulin was found to reach a maximal plateau level in 10 minutes. Since most clinical laboratories have a water bath for serological work available at this temperature, no special equipment is necessary. Thus, after the above preparations were removed from the ice water bath and after they reached room temperature, they were put into the water bath at 56°C for exactly 10 minutes. Fig. II gives the results obtained by this procedure with standard solutions of glucose and inulin. The color developed by inulin was linear to concentrations up to 25 μg.

Color formation by inulin was 83 times greater than that produced by an equivalent amount of glucose. Most important were the findings that the reaction on the part of glucose was constant and also that the color produced by glucose was additive to that produced by inulin. The observed analytic values of combinations of inulin and glucose corresponded closely to the calculated values as shown in the bottom of Table I. The recovery of unknown quantities of inulin added to varying known quantities of glucose has always been better than 98%. These facts permitted the use of a chromogenic blank for corrections in the analyses of inulin in blood and urine as has been done in other chemical procedures for inulin.

The method has been successfully applied for the micro-analysis of inulin in blood and urine using tungstic acid filtrates for plasma. Excellent agreement has been found with other chemical methods. This will be reported at the completion of our present work to allow the determination of both inulin and

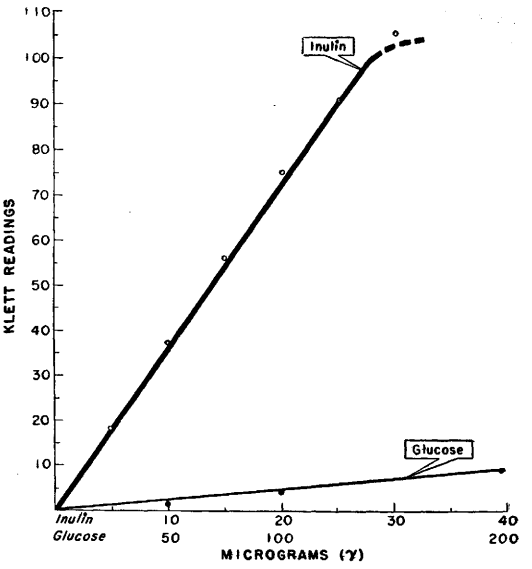

FIG. 2. Color formation by inulin and glucose with the "Indirect Technic." Color formation by inulin and glucose after heating with anthrone reagent in water bath 56°C for 10 min. Initial mixing of solutions with anthrone reagent was accomplished by eliminating significant heat of solution. Klett readings (λ = 620 μ) are given.

glucose in the same specimen of capillary finger blood by means of a chemical procedure similar to that reported here.

Summary. Use of the anthrone reagent offers a sensitive and accurate method that can be applied for the estimation of microquantities of inulin. Steps are given to prevent interference by glucose through the means of controlling the temperature of reaction. Boiling in alkali cannot be used to eliminate glucose because of an irregular distribution between blood and urine of the alkali labile fraction of inulin.

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Comparison of Blood Cell Counts from Major Vessels in the Dog.* (21098)

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Some investigators(1-3) have reported impressive differences in the number of cells found in blood taken from various sources in laboratory animals. However, other workers (4-6) have failed to confirm this observation. The present investigation was carried out on both normal and splenectomized dogs. Comparisons were made between samples obtained simultaneously from the aorta, hepatic vein, caudal vena cava, superior vena cava, and/or right ventricle in unanesthetized dogs and dogs anesthetized with pentobarbital sodium.

Methods. The catheterization of major arterial and venous vessels was performed in unanesthetized dogs by inserting small polyethylene catheters through a 15-gauge needle placed in the vessel after procaine infiltration of the area. The catheter was positioned by fluoroscopic visualization, the stylet removed and the catheter filled with 0.5% heparin in sterile saline. Catheters were placed in the aorta and in either the right ventricle or the superior vena cava. In addition to the comparisons with unanesthetized animals, a series of experiments was performed on animals under pentobarbital anesthesia (30 mg/kg body weight). In these experiments the catheters were placed in the right ventricle, and in the aorta and caudal vena cava at the anterior margin of the pelvic girdle. Additional data were accumulated from experiments in which Cournand catheters were placed in various positions in anesthetized and heparinized (5 mg/kg) animals, and in which all the catheters were introduced by exposing and ligating the vessels. In this series, comparisons were made with samples from the aorta and hepatic vein, and from the right ventricle and peripheral or hepatic vein. For the comparisons reported in this investigation data from samples drawn after any experimental procedure beyond that concerned with placing the catheters have not been included. The erythrocyte and leukocyte counts were done in duplicate, using National Bureau of Standard certified pipettes. The duplicate counts were required to check within 10%. At least 200 cells were counted in the differentials and checks were required between the first and second halves of the counts. Lymphocytes and monocytes were grouped together as mononuclear cells and no differentiation was made as to nuclear configuration of neutrophiles.

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