With development of the embryo, the strychnine spikes become of shorter duration and higher voltage, pointing to a better synchronization of the neurones. Frequency increases and spikes begin to appear earlier as the embryo grows. 6. From the 14th day spike discharges are to be seen in the embryonic brain, similar to the epileptic discharges produced in man and animals by certain agents like anoxia, strychnine, metrazol, and drying. 7. The bioelectric development is compared with the histological, cytological, and biochemical development of the brain, and with the physiological activity of the embryo.

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Interaction of Heparin with Auto-Agglutinins in Idiopathic Acquired Hemolytic Anemia.* (21096)

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Heparin was long considered the physiological regulator of the mechanism of blood coagulation(1,2). Later findings demonstrated its influence on plasma components belonging to the immune system, namely complement(3,4), and iso-agglutinins(5,6). It was also found that the antibody titer of rabbits sensitized against bacterial antigens increased manyfold upon previous or simultaneous administration of heparin(6). Astrup presented evidence which seemed to disprove the theory of heparin as a physiological anticoagulant. He showed that in no instance where "the tendency of blood to coagulate was greatly increased," could a rise in the blood heparin level be observed. This fact, with the other biological relations of heparin, has led him to advance the hypothesis that the "primary function of heparin in the organism should be looked for in connection with immunological processes"(7,8). Owren observed that the *in vivo* destruction of red blood cells in a patient suffering from acquired hemolytic anemia was retarded by large doses of heparin(9). Because of an increased bleeding tendency, the experiment had to be stopped after a few days, and this withholding of heparin resulted in a severe rise in the rate of cell destruction. No further comment as to the reason for using heparin and its possible mechanism of action was given, appar-

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TABLE I. Serological Findings in 3 Patients with Idiopathic Acquired Hemolytic Anemia.

Patient	Titer of Coombs test		Agglutinin titers*						Trypsin cells	Kolmer	Prothr. times†
			3°C		22°C		37°C				
	Direct	Indirect	Alb.	Saline	Alb.	Saline	Alb.	Saline			
R. R. (♀) Age: 17 A, CDEe/MN	1:128	1:2	1:1	1:1	1:8	Neg	1:32	Neg	1:1	Pos	17.2"
N. Y. (♀) Age: 35 O, cDEe/N	1:32	Neg	1:1	1:2	1:4	"	1:16	"	Neg	AC	14.5"
A. L. (♀) Age: 38 A, CDE . . .	1:256	"	Neg	1:4	1:64	1:2	1:64	"	1:1	Pos	16.2"

* The serial dilutions of serum were made using 0.1 patients serum plus 0.1 (20%) albumin or saline as a starting mixture. Same vol cell suspension was added to dilutions, incubated for 30 min. at temperatures listed, and subsequently centrifuged at 1000 rpm for 2 min.

† The normal prothrombin controls (100%) were 12.4" and 12.5".

ently because the experiment was "not wholly convincing . . ."

To our knowledge, the literature does not contain reports dealing with the interaction of heparin with auto-agglutinins. We would like, therefore, to present *in vitro* experiments, on 3 consecutive cases of idiopathic acquired hemolytic anemia, which appear to substantiate the possibility of such an interaction and to reemphasize the importance of heparin in immunologic processes.

Materials and methods. Commercial anti-human globulin serum (Ortho), bovine albumin (Armour), and Liquemin® (Heparin-Roche)† were used throughout. The concentrations of heparin employed ranged between 0.02-2.0 Toronto Units per 0.1 ml red blood cell suspension (5 x 10^5 cells). The characterization of the antibodies was performed by routine immunologic procedures. The cells were washed before performing the Coombs test, except where otherwise indicated. The cells of patient No. 3 were centrifuged only for the initial studies (600 rpm/one min.). In subsequent tests, the incubated mixtures were allowed to stand at room temperature for times ranging from 45 minutes to 6 hours. A "reverse" typing was necessarily performed on this patient, because of the almost immediate agglutination of her cells in any protein media. Heparin tolerance tests on plasma and whole blood were performed by the method of Waugh-Ruddick, as modified by Silverman (10). The diagnosis of iodiopathic acquired hemolytic anemia was made on the basis of severe anemia, reticulocytosis, elevated indirect serum bilirubin, spherocytosis, a positive direct Coombs test and the presence of auto-agglutinins in patients sera. Splenomegaly was present in all 3 cases; a careful survey revealed no apparent cause for the hemolytic process.

The studies here reported were made prior to any treatment or medication, except for one blood transfusion in patient No. 1. Patients Nos. 2 and 3 were relapse cases. Table I summarizes the serological findings of these patients on admission.

Results. When red blood cells from patient No. 1 and No. 2 were incubated with heparin for 10 minutes at room temperature and subsequently tested with anti-human globulin serum, it was found that they no longer agglutinated, thus giving a negative direct Coombs test. Coombs test titers performed with: a) heparin still present in the cell suspension, and b) removed by washing the incubated cells with fresh saline, gave the same results. Different concentrations of heparin

TABLE II. Effect of Heparin on Coombs Test Titer. Three patients.

Initial direct Coombs titer	Cells not washed after incubation with heparin	Cells incubated with heparin and subsequently washed				
		2.0 U	1.0 U	.5 U	.1 U	.02 U
1:128	Neg	Neg	Neg	Neg	1:1	1:8
1:32	"	"	"	1:1	1:4	1:32
1:256	"	See text . . .				

† "Organon" inc. Orange, N. J.

TABLE III. Effect of Heparin on Albumin Agglutination Titer.

Dilutions:		1:1	1:2	1:4	1:8	1:16	1:32	1:64
Serum diluted with albumin alone (control)	R. R.	4+	4+	3+	2+	2+	+	Neg
	N. Y.	4+	3+	2+	2+	+	Neg	"
As above, cells washed with heparin prior to incubation	R. R.	———	———	———	Neg	———	———	———
	N. Y.				"			
Serum diluted with heparinized albumin. (Heparin conc. constant)*	R. R.	———	———	———	Neg	———	———	———
	N. Y.				"			
Heparinized serum diluted with albumin. (Decreased heparin conc.)	R. R.	Neg	Neg	+	2+	2+	+	Neg
	N. Y.	"		2+	2+	+	Neg	"

* 10 units/ml.

affected the Coombs titer differently, and a rough parallelism between heparin concentration and decrease in titer could be observed, as shown in Table II.

In the case of patient No. 3, in order to avoid centrifugation, the cells were not washed after incubation with heparin. 0.1 ml anti-human globulin serum dilution was added to 0.2 ml incubation mixture (*i.e.*, 0.1 cells plus 0.1 heparin or saline) and read at room temperature. While visible agglutination could be detected (dil. 1:4) after only 2 minutes in the control, reaching the 1:64 dilution at 8 minutes, the tubes containing 0.5 unit of heparin required 15 minutes to show visible agglutination, and the reaction stopped after 30 minutes at 1:4. In the tubes with 1.0 unit of heparin, only a questionable agglutination developed after 45 minutes. Negative results were also obtained when washed cells from patients No. 1 and No. 2 were resuspended in albumin dilutions of their own serum, to which a constant amount of heparin was added, or when the cells were "washed" *with heparin* prior to incubation with serum and bovine albumin dilutions. However, in serial dilutions made with a heparinized serum (*i.e.*, decreasing heparin concentration in respect to the constant number of cells present), a delayed agglutination became visible (Table III).

Because of the difficulties encountered in handling the cells of patient No. 3, a different procedure was used in her case. From a test tube containing the patient's clotted blood, the clot was carefully removed and immersed in another tube containing the patient's clear serum. The resultant cell suspension was divided into 2 parts. Saline was added to one (0.2 ml) and the same volume of heparin (containing 10.0 U/cc serum) to the other. The samples were then left at room temperature for 6 hours. A heavy agglutination occurred in the tube containing the cell-serum suspension with saline after less than 15 minutes, whereas the heparinized suspension did not show visible agglutination at the end of the 6-hour period. Since certain observations to be discussed below seemed to indicate a direct interaction between heparin and the auto-agglutinins, it became of interest to determine whether heparin activity was impaired simultaneously with the loss of agglutination. It was observed that a heparin solution previously incubated with above patient's red blood cells lost much of its anticoagulant activity against normal plasma. For this reason heparin tolerance tests were performed on the plasma and whole blood of patients No. 1 and 2. The results, showing abnormally high heparin tolerances despite the elevated prothrombin times (Table I) are illustrated in Fig. 1. A normal control was performed only on whole blood.

Discussion. It becomes apparent that while the agglutinin titer decreases with increasing heparin concentrations, no correlation with the initial strength of the Coombs reaction can be established. The red blood cells from patient No. 2 require more heparin in order to become desensitized to the same extent as the cells of patient No. 1 (Table II), although she has a lower Coombs titer. This phenomenon is reemphasized in the heparin tolerance curves, where again the patient with the lower titer has the higher heparin tolerance (Fig. 1). Thus, there seems to be a qualitative difference between the agglutinins

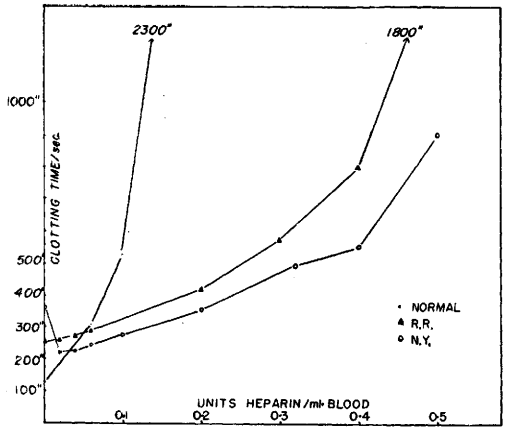

FIG. 1. Heparin tolerance test of two patients suffering from idiopathic acquired hemolytic anemia.

of these 2 patients, manifested by a difference in reactivity to heparin, and it is interesting to note that clinically the condition of patient No. 2 was more severe than that of No. 1. It is generally agreed that the Coombs reaction is no index of the gravity of a hemolytic episode. Whether the inhibition by heparin will prove to have any prognostic value awaits further accumulation of data. It was also shown that for the *in vitro* suppression of agglutination of coated red cells by antiglobulin serum or the patient's own serum, the presence of heparin is not necessary, provided the cells were previously incubated with it. From this follows that heparin does not inactivate or inhibit the antiglobulin serum or some "factor" in patient's serum, but reacts with the agglutinin itself. When one separates the cells previously incubated and washes them, the inhibition persists. At the same time, the cell-free heparin supernatant becomes almost inactive as an anticoagulant against normal plasma. Thus, the increased heparin tolerance of the blood could be due to the functioning of the agglutinin as a heparin inhibitor, at the expense of its own activity. Since heparin is closely related to hyaluronic acid (which was reported to yield upon depolimerization a distinctly anti-viral split product: glucuronolactone(11)), and possesses antithrombic and antihyaluronidase activity, similar to substances inhibiting competitively Newcastle disease and influenza virus hemagglutination(12), it could be included in the family of hemagglutinating-virus antagonists. The possibility of competition between auto-agglutinins and heparin should not be overlooked. However, the virus antagonists seem to inhibit agglutination *per se*, irrespective of the nature of the hemagglutinating factor, as evidenced by their action even on ABO and Rh antibodies(13). Heparin, on the contrary, does not seem to have any *in vitro* effect on isoagglutinins. Coombs tests performed on cells coated with iso-antibodies, (erythroblastotic cells and normal cells previously suspended in the serum of a patient having a 1:1024 anti-Rh titer), showed that treatment with heparin was ineffective in preventing agglutination‡(14). Therefore, whatever the mechanism of inhibition may prove to be, it seems specific for *auto*-agglutinins (or for a type of auto-agglutinins), and could become of diagnostic importance.

Concomitant with the inhibition of agglutination, a second reaction takes place, as suggested by the abnormal heparin tolerance curves (Fig. 1). Experiments to be reported showed that the prolonged prothrombin times of all 3 patients (Table I) were not due to a deficiency but to an inhibition. Using hyaluronidase(15) instead of heparin, a competition with the agglutinins for *proconvertin* was demonstrated. Such a competition, if valid also for heparin, may be the logical explanation for the initial correction of the recalcification time (patient N.Y.).

Finally, we would like to recall the relationship between proconvertin and complement, and the customarily encountered positive or anticomplementary Kolmer reactions (16) in acquired hemolytic anemia. The question arises whether this apparent "complexity" is but the manifestation of one and the same phenomenon, observed from different points of view; and that there need not be a contradiction between the old concept of Howell and the new concept expressed by Astrup in regard to the physiological role of heparin if one assumes that it acts on com-

‡ Valid also for cells sensitized *in vitro* with anti D (albumin) typing serum.

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