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# CHARACTERIZING GLUCOSAMINE INHIBITION OF THE RHESUS REACTION

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## INTRODUCTION

Writing in the Southern Medical Journal, in 1952, on the subject of the prevention of erythroblastosis fetalis, Diddle(16) commented that although the condition was known to Hippocrates and subsequent physicians, there had been no progress in the understanding of its pathogenesis or its treatment until 1940. Some important concepts had been formed before that time, however. Ballantyne, in 1892, according to Eastman(18), had established the illness as a definite clinical entity. Rautmann(16), in 1912, was impressed by the hematologic picture in the newborn and "appropriately" adopted the name of "erythroblastosis fetalis." Ottenberg(53) advanced a serologic explanation in 1923, attributing the pathology to a mixture of maternal and fetal blood factors; but he provided no evidence to corroborate his speculation. Darrow(14) reasoned similarly, suggesting that an actual antigen-antibody reaction was the etiology of 'erythroblastosis fetalis et neonatorum.' Moreover, eight years before this suggestion, Diamond, Blackfon, and Baty(15) had recognized that fetal edema, icterus

gravis neonatorum, and anemia of the newborn were pathogenetically part of a single process.

About 1940, a rapid succession of events established the true nature of this hemolytic disease. In that year Landsteiner and Wiener(44) reported the ability of some rabbit immune serum, produced in response to the blood of Rhesus monkeys, _Macaus rhesus_, to agglutinate 39 out of 45 (85 per cent) persons' sera, the agglutination not being explainable by any blood groups known at that time. One year previously Levine and Stetson(46) had reported the death following transfusion of a woman who previously had delivered a stillborn child. In this case report, they suspected that the patient had been immunized by an antigen from the dead fetus, the antigen in turn being inherited from the father. In 1940, Wiener and Peters(74) reported three fatal hemolytic reactions following transfusions of group compatible blood, noting that the same agglutinogen was present and responsible. In 1941, Levine, _et_. _al_., (47) and Landsteiner and Wiener(45) recognized the relation of Rh antigen to hemolytic disease of the newborn and outlined in general terms its isoimmunologic origin. The latter co-workers, by employing

a crude test to distinquish the Rh factor and by investigating family pedigrees, decided the factor was an autosomal dominant.

Gallagher and Jones(26) duplicated Landsteiner's initial results by injecting Rh positive erythrocytes into guinea pigs. They later showed that the anti-sera produced agglutinated cells not merely in an 85:15 ratio in Caucasians, but that the anti-sera agglutinated Rh positive cells only. Wiener and Belkin(2), employing agglutination-inhibition techniques, demonstrated that the Rh agglutinogen resided in the stroma of red cells and was a hapten in nature. Other investigators, including Zilliacus(78), Scott and others(67), and Clayton and others(11), had found fetal erythrocytes in the maternal circulation and thereby implicated their role in producing hemolytic disease of the newborn. Similarly, Levine and others(47), in 1941, had found the maternal agglutinin in fetal cord blood. These developments were aided significantly by the development of tests with enzymes and high molecular weight media to distinquish the Rh factor, but especially by the development by Coombs(12) of the anti-human globulin reaction.

PREVENTION OF ERYTHROBLASTOSIS FETALIS

## Significance of Erythroblastosis Fetalis

Statistically, of all marriages within Caucasian groups, and especially among Americans, only fifteen per cent are between Rh incompatible persons. The actual incidence of Rh hemolytic disease of the newborn, however, is estimated to be much less than fifteen per cent-- only about one to five per cent. The reasons for these discrepencies are many. To begin with, in only those marriages where the mother is Rh negative and the father Rh positive can the condition occur. Nevertheless, even to these parents only a five per cent incidence of erythroblastosis fetalis occurs because of other factors which are also operating. First-born infants are usually unaffected if Mother has previously not been sensitized, as, for example, by transfusion. Other blood group incompatibilities, for example, the ABO system, may interfere with Rh sensitization and preclude it. Finally, many couples, having had one or two catastrophes, will cease having children altogether. Implicit in this last statement, then, is the significance of erythroblastosis

fetalis and its prevention. When this condition strikes, it strikes the few; it usually strikes these same people repeatedly, leaving them childless or with only one child; and it occassionally renders would-be parents mental cripples. It is for the sake of these few couples, as well as for the yet unborn child, that there have been sought methods for reducing or modifying this disease.

Pathogenesis of Erythroblastosis Fetalis

While many exact details have yet to be elucidated, the general outline of pathogenesis of hemolytic disease is simple. To a Rh positive man and his Rh negative wife there is conceived a Rh positive child. During fetogenesis Rh positive cells escape from the fetal circulation, cross the placental barrier, and stimulate the production of anti-Rh antibodies (anti-Rh globulin). These antibodies, in turn, enter the fetal circulation, cause blood destruction, and produce hemolytic disease in any of its three clinical forms.

Much information is now extant to corroborate and enhance this skeleton outline. Clayton and others(11) in a series of 25 pre-partum patients found only four

per cent to have fetal erythrocytes in their circulation twenty-four hours before partuition. Post-partum, forty-eight per cent of these same patients demonstrated fetal red cells in their blood--revealing that the greatest portion of maternal transfusion occurs at the time of delivery and placental separation. By way of demonstrating that maternal factors do attack fetal cells, Zilliacus(78) found such fetal erythrocytes in maternal circulation which had been agglutinated. Jacobitz and Bryce(41) and Franklin and Kunkel(24) showed by centrifugation and electrophoretic techniques that pre-eminently it is only 7S gamma globulin (of which the blocking Rh factors are a part) which crosses the placenta, while the 19S gamma globulins, which contain the ABO system antibodies, do not. Moreover, as was pointed out by Wiener and Brancato(75), although generally the higher the titer of maternal antibodies, the greater is the frequency of stillbirths and neonatal deaths due to hemolytic disease, enough exceptions exist in both directions to cause the authors to speculate on the role of agents other than antibodies. Because of these exceptions and because even a Rh negative infant may effect an

anamnestic anti-Rh titer rise in the mother, Walker and Jellison undertook to predict neonatal involvement by spectrometric analysis of amniotic fluid as opposed to maternal titer--and, in their hands, with a greater degree of accurate correlation with clinical states(71).

## Prevention of Erythroblastosis Fetalis

Historically, the prevention of hemolytic disease of the newborn has taken many different directions, all directed against steps in the chain of pathogenesis, and all without noteworthy success to date(18). It has been suggested, for example, that incompatible marriages be prevented in order to preclude this disease; but because of the mores of the peoples, this suggestion is mentioned only in acknowledgment of the view or for the sake of completeness of the discussion; and then it is discarded. Different chemicals and reagents, including ascorbic acid, salicylates, and carbazochrome have been utilized to strengthen the capillary (placental) barrier and so prevent fetal-maternal exchange of both antigenic erythrocytes and resultant antibodies(11). However promising in theory, to date the results of such agents in actual practice have been disappointing(22).

Working on the basis of immunological antigenic suppression and on the fact that most Rh sensitization occurs during uterine trauma at the time of delivery, Freda and others(25) have shown that injecting into Rh negative males anti-Rh gamma globulin from twenty-four hours before to forty-eight hours after injecting Rh positive cells does, indeed, prevent Rh sensitization, at least as measured by antibody response on subsequent injection of Rh positive erythrocytes. However, because this gamma globulin is of the 7S fraction and will cross the placenta also, it cannot be used in the ante-partum state. According to Finn and others(21), moreover, any possible enhancement of the anti-D globulin which might occur by giving this gamma globulin can and is prevented by giving large doses of gamma globulin. Uhr and Bauman(70), utilizing guinea pigs, got results similar to Freda and others. They went further, however, showing that this inhibitory response was not effected by the presence or absence of complement, that suppression in general was inversely proportionate to the degree of dissociation of the antigen complex and to the strength of the antigen. They also found that because of antigen

dissociation *in vivo*, much more antibody was needed *in vivo* than *in vitro*.

Rh Hapten Inhibition of Erythroblastosis Fetalis

Possibly the most exciting work along the line of preventing erythroblastosis has been in the field of antibody inhibition where various substances, either isolated or discovered, appear or have appeared to prevent the antigen-antibody combination from occurring. Initial work along this line was performed in 1947 by Carter(7), following the leads of Belkin and Wiener(2) who reported having isolated the Rh factor from erythrocyte stroma, and Gallagher and Pillischer(27) who used this stroma to immunize guinea pigs. Carter, herself, injected the stroma intraperitoneally into guinea pigs and noted no anti-Rh response. Injecting the derivitive along with albumin, she found a mild anti-Rh response and interpreted this response as the result of "hapten plus protein carrier." In 1954(8), she reported on one hundred consecutive cases of Rh incompatible pregnancies where use of this hapten and protein averted abnormal infants. Unfortunately, she had no controls except arbitrary statistical ones; and her series was slanted by the inclusion of first-born infants and such, where hemolytic

disease tends to be precluded. In addition, she did not state whether or not her patients developed kernicteric indices. These facts are important since Zeitdin and Boorman(77) have shown that with maternal titers below 1:32 or 1:64 Coombs reaction, ninety-five per cent of cases need no help whatsoever. Diddle(16) found the hapten to be of no value in cases where the process was started when the patient first presented. Hamilton and Brockland(33), in a series of fifteen patients, found no reduction of antibody titer utilizing the Rh hapten; but their dosage magnitude and frequency were far smaller than that used by Carter. Subsequent use of this Rh hapten clinically has been disappointing(18). Significantly, Murray and others(52), reporting on hemagglutination-inhibition studies such as Carter had performed initially, tested the sedimentation rates of the involved cells and found that globulin was still on the cells. They proposed that while agglutination had indeed been inhibited, it was the aggregation phase and not the antigen-antibody reactive phase which had been involved.

Specific Inhibition of Erythroblastosis Fetalis

A mode of *in vitro* Rh inhibition similar to the Rh hapten found by Carter was introduced first by Selwyn(68) in 1949. He noted that Rh positive cells collected in "acid citrate" (disodium hydrogen citrate plus 2.5 per cent glucose) were not agglutinated by anti-D, anti-C, or anti-E antisera. Testing other blood group systems, he found that the ABO and MN systems were not affected. Selwyn stated that he ruled out pH as the cause and that the inhibitory effect seemed to him to be due to selective acid citrate absorption onto the erythrocyte surface, thereby preventing any reaction with the anti-Rh agglutinin. Unfortunately, no evidence for either of these statements was presented.

Nothing further along this line of specific anti-Rh inhibition was reported until Hackel's(30) work in 1958. Using a modified tube titer technique, he was able to show *in vitro* that the various ribonucleic acid derivitives were able to inhibit anti-Rh and anti-Lutheran sera--these derivitives being cytidine-sulfate, adenylic acid, uridylic acid, and cytidylic acid. Similar to Selwyn, Hackel found this inhibitory action

specific for the CDE system, but not the ABO, MN, He P, or $Fy^a$ systems. Attempting to further explore the specificity of the reaction, he noted that the constituents of the ribonucleic acid derivitives--D-ribose, purines, pyrimidines--revealed no such effect. In addition, in 1960(31), he treated Rh positive erythrocytes with ribonuclease, presumably to destroy the antigen factor, then noted that the titers of these cells fell when they were reacted with anti-C, anti-D, and anti-E antisera. The other blood group antigens mentioned previously remained unaffected.

Prager and others(61) using incubating techniques similar to Hackel found that acid phosphatase, alkaline phosphatase, and Russel Viper Venom had no depressing effect on titration scores, and that ribonuclease in about two-thirds the concentration used by Hackel had only reduced titration scores minimally. They also found, while checking other phosphate compounds, that phenolphthalein-disulfate, alpha-naphthyl phosphate, riboflavin-5'-phosphate, and alpha-tocopheral phosphate were all potent or complete inhibitors, their inhibition being maximum when the concentration of antiserum was

enough to agglutinate half the cells exposed. Their inhibitory effects were not considered specific, however, in view of the facts that other blood group systems were equally affected and that Coombs sera titers were not altered, indicating that perhaps the aggregation phase was involved.

In 1959, Boyd and others(6) reported on the specific inhibition of anti-D agglutinin by unnatural sugars--L-glucose, L-mannose, and D-gulose--and streptomycin, a natural glycoside of N-methyl-L-glucosamine which he suggested was the active portion. Pirofsky and Cordova(56) followed up Boyd's work by showing that not only was streptomycin sulfate an active inhibitor, but also dihydrostreptomycin sulfate, neomycin sulfate, paromomycin hydrochloride, pleocidin, streptidine, and dihydrostreptomycin with guanido groups hydrolyzed to amines. All these compounds contain hexosamine structures, and all were tested at 0.28 molar solution. At this concentration, N-acetyl-glucosamine and glucosamine hydrochloride were found to be totally ineffective--this result being in direct contrast to work done at this laboratory(54).

Dodd and others(17) found that not only did N-acetyl-neuraminic acid, ganglioside, N-acetyl-glucosamine, and N-acetyl-mannosamine inhibit the reaction between D positive cells and anti-D serum, but also that when these substances were adsorbed onto D negative cells, these cells were capable of adsorbing anti-D serum without subsequent agglutination. In addition, Bingley and Dodd(3), and Morgan(50), and Ceppillini(10)--all using conditioned hemagglutination experiments where red cells are modified by the adsorption of bacterial or plant substances onto their surfaces--found that such adsorbing substances would block anti-D sera specifically (of all the common blood group systems), and that the prime components of many of these factors were hexosamine (D-galactosamine, D-glucosamine) structures.

Hackel(32) recently reported that while after an optimum concentration was obtained, no further increase in 2'-adenosine-monophosphate would cause more anti-D serum inhibition, by adding histidine, more potent hemagglutination-inhibition was obtained. This finding, then, may represent an incipient synthesis to

his earlier work where he supposed the mode of inhibition to be neutralization of the antigen-antibody reaction secondary to the resemblance of the inhibitor to the antigen. Whereas Hackel(31) and Prager(61) had felt the phosphate or possibly the entire ribonuclease structure was essential, Boyd(6) and Dodd(17) had attributed this importance to a hexosamine or "complicated" hexosamine structure. It now appears from Hackel's work that both ribonucleic acid derivitives and amino acid structures might be effective, at least additively. Moreover, the notion of variable inhibitors for a "single" antibody is consistent with the well known facts that antibodies in general may be formed to various components of an antigen, and that anti-D serum activity is spread out by immunoelectrophoretic fractionation into many components(1). From the experiments on conditioned hemagglutination the possibilities exist that either there is steric hindrance by the inhibitor on the antibody near the antigenic locus or there is neutralization of the aggregation phase of the antigen-antibody reaction without any effect on the initial antigen-antibody phase.

ANTIGEN-ANTIBODY REACTIONS OF THE RHESUS SYSTEM

The remainder of this paper shall deal with personal investigative work which was carried out to characterize and determine the nature of this anti-Rh serum inhibition by specific substances. In view of our own success with D-glucosamine hydrochloride in inhibiting the Rh antibody, we have used this agent exclusively. Before discussing the methods and techniques used, it would seem imperative at this point to discuss the nature of the Rhesus antigen-anti-Rhesus antibody reaction.

Dynamics of the Rhesus Antigen-Antibody Reaction

The dynamics of the antigen-antibody reaction within the Rh system have been studied extensively by numerous investigators. Moving on the assumption that the reaction involved principles of mass action, Evans and others(19) showed that antibody could be transferred from sensitized Group A (D positive) cells to normal Group B (D positive) cells in accordance with such equilibrium, and that the pH, temperature, and incubation times were all factors in modifying the reaction. On the basis of this work and also investigations by Hughes-

Jones and others (vide infra), Weir(73) reasoned that co-valent bonds (a sharing of electrons) could play no part in the antigen-antibody reaction since they could not possibly dissolve as rapidly as the complexes have been found to do. Instead, Weir felt that complementary spacial arrangement of the reactive groups of the antigen and antibody allowed short range, low energy, inter-molecular forces to act and to keep the molecules together. These forces most likely are Coulomb forces, van der Wall forces, and hydrogen bonds. The validity for the van der Wall and Coulomb forces, which are attraction forces between positive and negative charged groups, is found partially in the dissociation of the bonds in acidic and alkaline solutions, and also in highly ionic solutions where ions may compete with the charged areas on the antigens and antibodies and prevent their reaction. Weir*s hypothesis is also consistent with the facts that to date most of the compounds which have been found to inhibit the anti-Rh reaction contain either amine or phosphate radicals, both of which chemically are "electron rich" compounds and may dissociate in solution of offer electron

or steric hindrance to antigen-antibody association. Or, if one agrees with Weinbach(72) who believes an antigen-antibody reaction is really the formation of electron donor and electron acceptor complexes, inhibition might be due to the fact that the inhibitor accepts the electrons offered by the antibody donor and so prevents the initial antigen-antibody reaction from occurring.

Again utilizing $I^{131}$-tagged anti-D serum, Evans and others(20) found that even after clumping had occurred, non-tagged antibody in excess was able to displace tagged anti-D from the agglutinated red cells, and that anti-D was adsorbed maximally onto -D-/-D- cells, least onto cde/cde cells, and somewhat less in each group where the C antigen was present. Using tagged anti-D in a similar fashion, Renton and Hancock(65) showed that incomplete (albumin) anti-D serum blocked complete (saline) anti-D serum inversely proportionate to the potency of the antigen, this potency being ranked in descending order as follows: -D-/-D-, cDE/cDE, ---/-D-, $D^u$, D-negative.

In a series of investigations utilizing $I^{131}$-labeled anti-D serum, Hughes-Jones and others found

that the association constant of the reaction was heterogeneous to numerous determinations (values ranged from 1.2 to 1.8 x $10^8$ L/mole), that the energy of activation of the antigen-antibody complex was 13,500 Cal/ $^{o}$C/mole, that the bond strength between the antigen and antibody increased up to 37$^{o}$C, that the rate of association of antigen and antibody was proportional to increasing temperature, this phenomenon being secondary to decreased fluid viscosity(37). Unlike Evans who assumed a monomolecular reaction occurred throughout the reaction, Hughes-Jones felt that the associative reaction was a bimolecular one and the dissociative reaction, a monomolecular one(36)(37)--but neither man gave reasons for his opinions. In other experiments Hughes-Jones found that antigen-antibody association is maximum at a pH of 6.5 to 7.5, with a more acidic environment giving greater dissociation and a more alkaline environment promoting dissociation and hemolysis(40). The mechanism of action was felt not to be a direct one on the complex, but rather on the antibody configuration; but, again, no evidence was discussed. Decreased ionic strengths of suspending media, in general, and of sodium chloride

specifically were found to enhance significantly the associative phase of the Rhesus antigen-antibody reaction(39)(40). From all this, then, Hughes-Jones concluded that the optimum conditions for detecting Rh group antibodies by simple reactions and antiglobulin techniques were those where the pH was 6.5 to 7.5, the temperature was 37°C, the saline molarity was 0.034 molar, and the incubation time was 15 to 30 minutes(40).

Rhesus Antigen

Information on the Rhesus antigen is scanty. From the work of Hackel(31), Pirofsky and Cordova(56), and Dodd(17), it is felt to contain an essential phosphate or amine, or glucose moiety--or all three. From the $I^{131}$-tagged anti-D sera studies of Hughes-Jones(35) or the quantitative hemagglutination studies of Evans(20) and Rosenfield(66), the antigenic sites or loci per erythrocyte is estimated variously from 10,000 to 25,000 to 68,000. And on the basis of numerous enzyme, antihuman globulin, and elution studies (*vide infra*), the sites are felt to be located at the cell surface or in its stroma(39)(7)(27).

Rhesus Antibody

The anti-Rh globulins have been studied by various techniques since Race(62) first discovered the incomplete antibodies which prevent saline anti-Rh agglutination. These methods include ultracentrifugation, electrophoresis, sedimentation analysis, and serology. Kerwick and Mollison(43) utilized all four in analyzing the sera of nine patients and found that "anti-D" was composed primarily of 7S gamma globulin, with less than one per cent being 19S gamma globulin. Like all 7S gamma globulin, incomplete anti-Rh was found to have a molecular weight of about 160,000 AWU, a length of 270Å.U., and a width of 37Å.U. The 19S saline anti-Rh had a molecular weight of 1,000,000 AWU, a length of 900Å.U., and a width of 37Å.U. Abelson and Rawson(1) used chromatographic techniques to show that anti-D serum could be separated from ABO serums by numerous differences, and they also pointed out that the broad bands of incomplete antibody and the sharp bands of complete anti-Rh which they found were consistent with the notions that incomplete antibodies were heterogeneous collections while saline ones were

more homogeneous. These authors felt from their brief studies on Rh hemolytic disease that initial immunization occurs with saline ($gamma_1$ M fraction) antibodies and that further immunization occurs with 7S blocking (gamma SS fraction) antibodies. By serologic techniques(64) they found that papain-digested gamma SS anti-Rh serum yielded 'S' and 'F' fractions, and that an anti-F serum (prepared similar to anti-gamma SS or Coombs serum) precipitated all incomplete antibodies detected by Coombs serum. Anti-S serum also produced this phenomenon but was more apt to cause prozone. Franklin and Fudenberg(23), using electrophoretic and ultracentrifuge techniques to analyze various globulin agents, found that the Rh immune globulins, as those of any immune disease, consisted of light chains divisible into subgroups I(A) and II(B). Seven S gamma globulin of Rh hemolytic disease was found to be sixty per cent I and forty per cent II. Moreover, papain, pepsin, and other enzyme digestions broke these entities down to the 3.5S, 5S, and such fractions, again definitely pointing out their heterogeneity.

The ability of certain techniques for enhancing

the agglutination of Rh positive cells by albumin anti-D has long been known: 1) increased centrifugation by enhancing proximity of cells and overcoming circumferential ionic field forces(69), 2) incubation of antibody with mercaptoethanol presumably to change the tertiary structure of albumin anti-D to a saline form(55), 3) prolonged incubation by permitting anti-D more time to react, 4) enzymes (papain, trypsin, bromelin, ficin, and others) by uncovering or erecting sites on the antigen or antibody(76) or by entropic energy release during the antigen-antibody reaction(60), and 5) high molecular weight media (albumin) presumably by changing or rearranging surface molecular forces(59). As Goodman and Masartis(28) point out, all these procedures point up the fact that incomplete anti-D is not truly univalent since mechanical techniques and red cell modifying techniques should not increase agglutination titers if this were the case. Moreover, the generally held belief that albumin anti-D is made from larger saline anti-D molecules or vice versa cannot hold since in rabbits saline antibody is generally smaller and yet the same sets of statistics for enzymes and high

molecular weight media hold there also. Goodman and Masartis believe that the strengthening of already existing bonds, either by papain treatment of cells or by the addition of anti-human globulin is the factor inducing the agglutination phenomenon.

## EXPERIMENTAL INVESTIGATION

In our own laboratories(54), the work of Hackel and others had been repeated, and in contrast to Pirofsky and Cordova(56), and Boyd(6) two new agents--D-glucosamine hydrochloride and pyridoxal phosphate--were found to have significant *in vitro* inhibitory ability. In order to gain competent experience in the technique, the in vitro work of Pearse and Hobel with glucosamine was repeated, and numerous variations were performed to characterize the reaction. It was found that under wide ranges of pH and osmolality (concentration) glucosamine had an inhibitory effect on both saline and albumin type Rh antibodies, that this effect was to a certain extent proportional to the concentration of the glucosamine, that it was independent of molecular size or osmolality of the solution *per se*, and that its

effect appeared to be on the erythrocyte surface and not on the antibody itself.

<u>General Methods</u>

The overall technique was as follows: The solutions-to-be-tested were prepared in various manners, according to the pattern or the objective to be tested. For titration of the Rh agglutinins, two-fold dilutions were carried out with 0.85 per cent sodium chloride or twenty-two per cent bovine albumin, as was appropriate, employing the clean multiple pipettes method. Then, .05 ml. of the test solution was incubated with .05 ml. of each antisera titer at 37°C for thirty minutes, with intermittent mixing. Following this incubation, .05 ml. of a two per cent Group O (D positive) erythrocyte suspension in either 0.85 per cent sodium chloride or twenty-two per cent bovine albumin, as appropriate, was added; and the total mixture was then incubated at 37°C for sixty minutes with intermittent mixing. The mixture was then centrifuged at 1500 RPM for one minute, shaken gently, and the degree of agglutination was read by the Race Scale (Table I)(63). Negative controls to rule out pan-agglutination

## TABLE I
## Race Scale for Agglutination

| Description | Score |
|---|---|
| Agglutination clearly visible to naked eye. . . . | 10 |
| Very large aggregates seen microscopically. . . . | 8 |
| Large agglutinates seen microscopically . . . . . | 5 |
| Small agglutinates seen microscopically . . . . . | 3 |
| Smallest definite agglutinates seen . . . . . . . | 2 |
| No agglutination seen, cells evenly distributed . | 0 |

or such similar phenomena were run in all cases, utilizing either Rh negative erythrocytes instead of Rh positive ones, or twenty-two per cent bovine albumin instead of anti-D serum. Positive controls were run utilizing normal saline instead of test solutions. Before using any antiserum of commercial preparation, the serum was heated to 56°C for sixty minutes to inactivate complement. In all cases dilutions were expressed as titers, and final hemagglutination results were given in Race scores.

<u>An Example of Data Interpretation</u>

An example of the use of the Race score to quantitate agglutination follows: One may suppose that $C_1$ is a negative control, consisting of D positive cells, bovine albumin and saline in equal quantities. No agglutination would be expected, even microscopically; and the total Race score if none were found, would be "0." Similarly, $C_2$ is a positive control consisting of anti-D serum in various dilutions, D positive cells, and saline instead of test solutions. Agglutination would be expected in all dilutions, and in our example, it occurs. However, for various reasons

inherent in a technique with large numbers of samples, as well as with aging antiserum or cells, agglutination may not be visible grossly, yet still occur under the microscope. Titers 128, 256, and 512 of $C_2$ are read in this example as showing decreased agglutination. $T_1$ consists of a test solution (A) which was incubated with anti-D, then the mixture was reacted with D positive cells under the standard procedure outline previously. A minimal reduction of Race score may be noted, but unless the amount of reduction is significant (at least twenty to thirty Race points or two tubes gross agglutination) valid conclusions concerning the inhibitory potency of the test agent are not possible. The reason is that by repeating numerous tests with the same solutions of anti-D, glucosamine, and red cell suspensions a Race score difference of up to fourteen points occurred--this amount representing the variation in our technique. $T_2$ consists of test solution B, anti-D serum, and D positive cells treated in the usual manner. A significant reduction in Race score of forty-four points is noted and indicates that a mechanism of inhibition is operative. All this is summarized in Table II.

**TABLE II**

**Example of Data Tabulation**

$C_1$: D pos cells, albumin, saline
$C_2$: D pos cells, anti-D serum, saline
$T_1$: D pos cells, anti-D serum, test solution A
$T_2$: D pos cells, anti-D serum, test solution B

| Dilution | (Titer) | $C_1$ | $C_2$ | $T_1$ | $T_2$ |
|---|---|---|---|---|---|
| 1:1 | 1 | 0 | 10 | 10 | 10 |
| 1:2 | 2 | 0 | 10 | 10 | 10 |
| 1:4 — 1:8 ? | 4 | 0 | 10 | 10 | 8 |
| 1:16 | 16 | 0 | 10 | 10 | 5 |
| 1:32 | 32 | 0 | 10 | 10 | 2 |
| 1:64 | 64 | 0 | 10 | 8 | 2 |
| 1:128 | 128 | 0 | 8 | 8 | 0 |
| 1:256 | 256 | 0 | 8 | 5 | 0 |
| 1:512 | 512 | 0 | 5 | 0 | 0 |
| Total | | 0 | 91 | 81 | 47 |
| | | | 81 | 71 | 37 |

It should be obvious at this point, or at least should be pointed out, that the Race score is meant only to compare degrees of agglutination, and it is in no way meant to be more than grossly quantitative. Thus, it would not be valid to say in this experiment that fifty per cent of the anti-D antibody had been neutralized by test solution B since the Race score was reduced by one-half. Indeed, as will be discussed later, possibly none of the antibody had been neutralized. However, it would seem accurate to say that agglutination had been prevented.

## Preparing Test Solutions

Ten per cent solutions of glucosamine-hydrochloride (formula weight of 216.5 grams) were prepared by dissolving one gram of glucosamine-hydrochloride initially in nine milliliters of 0.2 M Sorensen's phosphate buffer. The pH of the solution, which was always acidic (about pH of 4.2), was adjusted to 7.0 by the addition of 0.1 M potassium hydroxide. Enough phosphate buffer was then added to fill the total volume to ten milliliters. Subsequent dilutions of the glucosamine were made using 0.2 M phosphate buffer.

Saline controls for the phosphate buffer were set up using glucosamine-hydrochloride dissolved in 0.85 per cent sodium chloride; and plain controls were established by dissolving the glucosamine in triple distilled water. In all cases the pH of the final solution was adjusted to between 6.8 and 7.4 with 0.1 M potassium hydroxide.

Effect of Osmolality on Glucosamine Inhibition

Despite variations in the osmolality of the glucosamine solutions prepared in phosphate buffer (osmolality of 752 mOsm/L), in normal saline (osmolality of 640 mOsm/L), and in triple distilled water (osmolality of 428 mOsm/L) as measured by the Advanced Osmometer, results of agglutination-inhibition tests were remarkably similar for all concentrations of glucosamine tested. That is, agglutination did not differ by more than one tube gross agglutination or ten Race points. Moreover, any change in erythrocytes noted for one solution was noted for all the others. The reason for the similarity of final results despite variation in osmolality and despite the fact that Hughes-Jones found that osmolality to either side

of 280 mOsm/L depressed the associative phase of the Rh antigen-antibody reaction, may lie in the fact that when quantities of the various ten per cent solutions were mixed with equal quantities of anti-D serum and two per cent erythrocyte suspensions, the final osmolalities of all the total mixtures were remarkably similar: 332 mOsm/L for glucosamine in triple distilled water, 358 mOsm/L for glucosamine in normal saline, and 380 mOsm/L for glucosamine in phosphate buffer. In addition, the grossness of our techniques compared to that of Hughes-Jones and others(40) explains, to some extent, our failure to find small variations with osmolality. In view of our findings, it was decided to use 0.2 M Sorensen's phosphate buffer as our diluent. In addition, because of similar findings for albumin and saline concentrations within physiologic ranges, it was decided to use 0.85 per cent sodium chloride and twenty-two per cent bovine albumin, as appropriate, for diluting anti-sera and for preparing red cell suspensions.

Because of the findings of Pearse and Hobel(54) that nearly all agents, including glucose and buffer

salts, would inhibit incomplete (albumin) anti-D agglutination *in vitro,* we have used saline anti-D almost exclusively in testing the effect of various parameters on glucosamine inhibition.

Effect of pH on Glucosamine Inhibition

The effect of pH on glucosamine inhibition was tested by preparing a five per cent solution of D-glucosamine hydrochloride/0.2 M phosphate buffer, and adjusting the pH from 3.4 to 8.2 by increments of four-tenths pH units, utilizing 0.1 M hydrochloric acid and 0.1 M potassium hydroxide. All pH reactions were read by pHydrion test papers graded to two-tenths pH units. In all other respects--dilution titers, erythrocyte suspensions, incubations, and centrifugations--the solutions and reagents were handled in the standard manner. Typical results are shown in Table III.

From this table it would appear the glucosamine inhibition is specifically enhanced outside the pH levels of 6.0 to 7.8. In view of the fact that Hughes-Jones and others(38)(40) had demonstrated that extremes of pH do lower the association constant of D cells and anti-D serum, it was felt worthwhile to repeat this

## TABLE III

### Glucosamine Inhibition with pH Variation

| Titer / pH of solution | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3.4 | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | --- |
| 3.8 | 10 | 3 | 2 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 20 |
| 4.2 | 10 | 10 | 2 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 27 |
| 4.6 | 8 | 5 | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 18 |
| 5.0 | 10 | 8 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 23 |
| 5.4 | 10 | 10 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 22 |
| 5.8 | 10 | 10 | 10 | 5 | 5 | 5 | 0 | 0 | 0 | 0 | 45 |
| 6.2 | 10 | 10 | 10 | 8 | 5 | 0 | 0 | 0 | 0 | 0 | 43 |
| 6.6 | 10 | 10 | 10 | 10 | 3 | 2 | 0 | 0 | 0 | 0 | 45 |
| 7.0 | 10 | 10 | 10 | 5 | 8 | 0 | 0 | 0 | 0 | 0 | 55 |
| 7.4 | 10 | 10 | 10 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 35 |
| 7.8 | 10 | 10 | 8 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 36 |
| 8.2 | Lysis | Lys | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | --- |

TABLE IV

Glucose Inhibition with pH Variation

| Titer / PH of solution | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3.4 | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | --- |
| 3.8 | 10 | 10 | 5 | 8 | 5 | Lysis | 0 | 0 | Lysis | Lysis | 38 |
| 4.2 | 10 | 10 | 10 | 5 | 2 | 2 | 0 | 0 | 0 | 0 | 39 |
| 4.6 | 10 | 10 | 5 | 8 | 2 | 0 | 0 | 0 | 0 | 0 | 45 |
| 5.0 | 10 | 10 | 10 | 8 | 8 | 5 | 0 | 0 | 0 | 0 | 51 |
| 5.4 | 10 | 10 | 10 | 10 | 8 | 5 | 0 | 0 | 0 | 0 | 53 |
| 5.8 | 10 | 10 | 10 | 10 | 10 | 10 | 2 | 2 | 0 | 0 | 64 |
| 6.2 | 10 | 10 | 10 | 10 | 10 | 10 | 8 | 8 | 5 | 2 | 83 |
| 6.6 | 10 | 10 | 10 | 10 | 10 | 10 | 5 | 5 | 2 | 0 | 72 |
| 7.0 | 10 | 10 | 10 | 10 | 10 | 10 | 8 | 5 | 2 | 2 | 77 |
| 7.4 | 10 | 10 | 10 | 10 | 10 | 10 | 5 | 5 | 2 | 2 | 74 |
| 7.8 | 10 | 10 | 10 | 10 | 8 | 3 | 5 | 0 | 2 | 0 | 58 |
| 8.2 | 5 | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | Lysis | --- |

experiment, utilizing solutions of five per cent glucose/ 0.2 M phosphate buffer, prepared and treated exactly as was the five per cent glucosamine/0.2 M phosphate solution. The results here could then be compared with those of glucosamine. This procedure was carried out, and the results are shown in table IV.

Although the agglutination titers were, on the whole, much higher for a given pH value of glucose than for the same pH in glucosamine, the general trend is similar, Namely, there is a distinct depression of hemagglutination below pH of 5.8 to 6.0 and above pH of 7.8 to 8.0. At these pH levels the dissociation constant of the Rhesus antigen-antibody reaction predominates over the associative one, according to Hughes-Jones and others(38) and there is an increase of like surface charges with subsequent repulsion of cells, according to Evans(19). In either event, the enhancement of inhibitory power of glucosamine over the Rhesus reaction by extremes of pH appears to be non-specific in its nature.

Effect of Time on Glucosamine Inhibition

Since both Hughes-Jones and others(37) and Evans(20) had pointed out the dissociative character of the Rhesus antigen-antibody reaction, it was felt necessary to determine whether time variations had any effect on agglutination titers or agglutination-inhibition titers. Also, it was not known whether a pre-incubation time was needed. To secure the answers to these problems, the following experiments were performed. A five per cent glucosamine-hydrochloride/0.2 M phosphate buffer solution was prepared in the usual manner. A negative control was established using albumin; an agglutination positive control using saline instead of glucosamine; an agglutination inhibitory control using glucosamine with incubation times of thirty minutes for reaction with anti-D serum and sixty minutes for reaction with red cells. In test A the second incubation was carried out for 120 minutes; in test B, for 240 minutes; in test D, for 1440 minutes. In test E the initial incubation was 20 minutes; in test F, 10 minutes; in test G, 1 minute. In test H the glucosamine was added to the D positive erythrocytes 3 minutes after

the anti-D serum was added. Results of these time variations are shown in Table V.

From this table which represents only a few experiments certain trends are noted. Once inhibition occurs (tests B,C, and D), it persists. This indicates that the glucosamine inhibitory action is not transient. On the other hand, once the antigen-antibody reaction occurs, it persists. Despite the fact that the antigen-antibody reaction is an associative and dissociative one, the latter phase occurs too slowly, at least over a twenty-four hour period, to be noted by our test methods or to give the appearance of an enhanced inhibitory effect of glucosamine. Both these findings of persistent inhibition once inhibition had occurred and of persistent agglutination once agglutination had occurred, as well as the trend toward increased agglutination with decreased antibody-glucosamine incubation time, could be explained 1) on the basis of a competitive inhibition phenomenon where the antibody would become firmly attached to the first "hapten," either red cell or glucosamine, with which it came in contact, or 2) on the basis of red cell surface alteration where once the antigen-antibody

## TABLE V

### Glucosamine Inhibition with Time Variations

$C_1$: D pos cells, albumin, saline, incubation of 30-60 minutes
$C_2$: D pos cells, anti-D serum, saline, incubation of 30-60 minutes
$C_3$: D pos cells, anti-D serum, glucosamine, incubation of 30-60 min.
A : Same agents as $C_3$, incubation of 30-120 minutes
B : Same agents as $C_3$, incubation of 30-240 minutes
D : Same agents as $C_3$, incubation of 30-1440 minutes
E : Same agents as $C_3$, incubation of 20-60 minutes
F : Same agents as $C_3$, incubation of 10-60 minutes
G : Same agents as $C_3$, incubation of 1-60 minutes
H : Same agnets as $C_3$, incubation of "reverse 3"-60 minutes

| Titer | $C_1$ | $C_2$ | B | A | $C_3$ | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| 2 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| 4 | 0 | 10 | 10 | 5 | 5 | 8 | 10 | 10 | 10 | 10 |
| 8 | 0 | 10 | 3 | 3 | 2 | 8 | 8 | 10 | 10 | 10 |
| 16 | 0 | 10 | 2 | 0 | 0 | 5 | 5 | 8 | 8 | 10 |
| 32 | 0 | 10 | 0 | 2 | 0 | 2 | 5 | 2 | 8 | 10 |
| 64 | 0 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 5 |
| 128 | 0 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 216 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| 512 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Total | 0 | 86 | 35 | 28 | 27 | 43 | 48 | 50 | 61 | 75 |

reaction occurred, glucosamine could no longer affect it.

Effect of Concentration on Glucosamine Inhibition

Since our results with D-glucosamine hydrochloride as an inhibitor of the anti-Rhesus reaction were almost diametrically opposed to the reports of Pirofsky and Cordova(56) and Boyd(6) who found that this agent did not work in concentrations of 0.28 molar and 0.25 molar solutions respectively, it was decided to test the effect of various concentrations of D-glucosamine as inhibitors. To do this, a series of glucosamine solutions ranging from one per cent to ten per cent were prepared in 0.2 M Sorensen's phosphate buffer in the usual manner; and studies were carried out as described previously. A one per cent glucosamine hydrochloride solution is 0.046 molar. Empirical osmolalities of the solutions were determined on the Advanced Osmometer:

1% . . . . . . . . 210 mOsm/L
2% . . . . . . . . 307 mOsm/L
3% . . . . . . . . 402 mOsm/L
4% . . . . . . . . 487 mOsm/L
5% . . . . . . . . 536 mOsm/L
6% . . . . . . . . 608 mOsm/L
7% . . . . . . . . 618 mOsm/L
8% . . . . . . . . 723 mOsm/L
9% . . . . . . . . 754 mOsm/L
10% . . . . . . . 757 mOsm/L

Results of this experiment are shown in table VI(A).

## TABLE VI(A)

### Glucosamine Inhibition with Varying Concentrations

Solution A: D pos cells, albumin, saline
Solution B: D pos cells, anti-D serum, saline
Solution C: D pos cells, anti-D serum, 1% glucosamine
Solution D: D pos cells, anti-D serum, 2% glucosamine
Solution E: D pos cells, anti-D serum, 3% glucosamine
Solution F: D pos cells, anti-D serum, 4% glucosamine
Solution G: D pos cells, anti-D serum, 5% glucosamine
Solution H: D pos cells, anti-D serum, 6% glucosamine
Solution I: D pos cells, anti-D serum, 7% glucosamine
Solution J: D pos cells, anti-D serum, 8% glucosamine
Solution K: D pos cells, anti-D serum, 9% glucosamine
Solution L: D pos cells, anti-D serum, 10% glucosamine

| Titer | A | B | C | D | E | F | G | H | I | J | K | L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 5 | 5 | 0 | 0 |
| 2 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 8 | 3 | 0 | 0 | 0 |
| 4 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 5 | 3 | 0 | 0 | 0 |
| 8 | 0 | 10 | 10 | 10 | 10 | 10 | 5 | 3 | 0 | 0 | 0 | 0 |
| 16 | 0 | 10 | 10 | 8 | 8 | 5 | 3 | 0 | 0 | 0 | 0 | 0 |
| 32 | 0 | 10 | 8 | 5 | 5 | 2 | 2 | 0 | 0 | 0 | 0 | 0 |
| 64 | 0 | 5 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 128 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 256 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 512 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 0 | 72 | 60 | 53 | 53 | 49 | 40 | 26 | 11 | 5 | 0 | 0 |

TABLE VI(B)

Race Score Following Coombs Testing of Solutions in Table VI(A)

| Titer | A | B | C | D | E | F | G | H | I | J | K | L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 5 | 0 | 0 |
| 2 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 0 | 0 | 0 |
| 4 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 5 | 3 | 0 | 0 | 0 |
| 8 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 3 | 0 | 0 | 0 | 0 |
| 16 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 0 | 0 | 0 | 0 | 0 |
| 32 | 0 | 10 | 10 | 10 | 10 | 10 | 10 | 0 | 0 | 0 | 0 | 0 |
| 64 | 0 | 10 | 10 | 10 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| 128 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 256 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 512 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Totals | 0 | 100 | 70 | 70 | 60 | 70 | 60 | 28 | 23 | 5 | 0 | 0 |

Inspection of this data reveals that the increase in concentration of the glucosamine is associated with increased inhibitory action, and that this increase in inhibition does not parallel the increase in osmolality of the total solution. From these results it would appear that the failure of Pirofsky and Cordova and of Boyd to elucidate this inhibitory activity of glucosamine was on a dosage basis, their doses being just below the concentration for a noteworthy effect with the concentrations of anti-D serum which they used. Pearse and Hobel (54) reported on the apparent failure of 1000 ml of ten per cent glucosamine injected intravenously into a patient with a rising Rhesus titer to reduce the titer significantly; and if, as they estimated, the achieved concentration of glucosamine was two per cent, then our lack of effect at this concentration *in vitro* correlates well with theirs *in vivo*.

Universality of Glucosamine Inhibition

D-glucosamine inhibition in ten per cent concentration was tested against anti-A, anti-B, anti-M, anti-N, anti-C, anti-c, anti-E, and anti-e antisera, commercially produced by Dade Laboratories. For each

antiserum negative and positive controls were performed, and in each study 0.05 ml quantity of reagent was used. The procedure was carried out in the usual manner, and the results are to be found in Tables VII(A), VII(B), and VII(C).

The results of this experiment indicate that glucosamine is specific, so far as this study was carried, for the Rhesus without any effect on the ABO and MN systems. This finding is similar to those findings of Selwyn(68) who worked with "acid citrate," to those of Hackel(31) who worked with ribonucleic acid derivitives, and to those of Boyd(6) who worked with streptomycin. These workers found that the inhibitory power of their agents did not extend to the ABO and MN systems, also. While it is tempting to generalize, as did Selwyn, Hackel, and Boyd, that the inhibitor (in our case, glucosamine) is specific for the Rhesus system and so must be related to the specific structure of the Rhesus determining groups, such considerations may not be the actual case. It may be possible, for example, that glucosamine alters the cellular surface near the locus of the antigen without affecting the

## TABLE VII(A)

### Inhibitory Range of Glucosamine

Solution A: Group A cells, albumin, saline
Solution B: Group A cells, anti-A serum, saline
Solution C: Group A cells, anti-A serum, glucosamine
Solution D: Group B cells, albumin, saline
Solution E: Group B cells, anti-B serum, saline
Solution F: Group B cells, anti-B serum, glucosamine

| Titer | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| 1 | 0 | 10 | 10 | 0 | 10 | 10 |
| 2 | 0 | 10 | 10 | 0 | 10 | 10 |
| 4 | 0 | 10 | 10 | 0 | 10 | 10 |
| 8 | 0 | 10 | 10 | 0 | 10 | 10 |
| 16 | 0 | 10 | 10 | 0 | 10 | 10 |
| 32 | 0 | 10 | 10 | 0 | 10 | 8 |
| 64 | 0 | 3 | 5 | 0 | 8 | 5 |
| 128 | 0 | 3 | 2 | 0 | 8 | 5 |
| 256 | 0 | 2 | 0 | 0 | 5 | 0 |
| 512 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 0 | 68 | 67 | 0 | 83 | 78 |

## TABLE VII(B)

### Inhibitory Range of Glucosamine

Solution G: Group MN cells, albumin, saline
Solution H: Group MN cells, anti-M serum, saline
Solution I: Group MN cells, anti-M serum, glucosamine
Solution J: Group MN cells, anti-N serum, saline
Solution K: Group MN cells, anti-N serum, glucosamine

| Titer | G | H | I | J | K |
|---|---|---|---|---|---|
| 1 | 0 | 10 | 10 | 10 | 10 |
| 2 | 0 | 10 | 10 | 10 | 10 |
| 4 | 0 | 10 | 10 | 8 | 8 |
| 8 | 0 | 10 | 10 | 5 | 8 |
| 16 | 0 | 5 | 3 | 2 | 5 |
| 32 | 0 | 2 | 2 | 0 | 2 |
| 64 | 0 | 0 | 2 | 0 | 2 |
| 128 | 0 | 0 | 0 | 0 | 0 |
| 256 | 0 | 0 | 0 | 0 | 0 |
| 512 | 0 | 0 | 0 | 0 | 0 |
| Total | 0 | 47 | 47 | 35 | 45 |

TABLE VII(C)

Inhibitory Range of Glucosamine

Solution L: Group C cells, albumin, saline
Solution M: Group C cells, anti-C serum, saline
Solution N: Group C cells, anti-C serum, glucosamine
Solution O: Group c cells, albumin, saline
Solution P: Group c cells, anti-c serum, saline
Solution Q: Group c cells, anti-c serum, glucosamine
Solution R: Group E cells, albumin, saline
Solution S: Group E cells, anti-E serum, saline
Solution T: Group E cells, anti-E serum, glucosamine
Solution U: Group e cells, albumin, saline
Solution V: Group e cells, anti-e serum, saline
Solution W: Group e cells, anti-e serum, glucosamine

| Titer | L | M | N | O | P | Q | R | S | T | U | V | W |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 10 | 0 | 0 | 10 | 8 | 0 | 10 | 8 | 0 | 10 | 10 |
| 2 | 0 | 10 | 0 | 0 | 10 | 2 | 0 | 10 | 5 | 0 | 10 | 5 |
| 4 | 0 | 10 | 0 | 0 | 10 | 0 | 0 | 10 | 0 | 0 | 10 | 2 |
| 8 | 0 | 5 | 0 | 0 | 8 | 0 | 0 | 10 | 0 | 0 | 10 | 0 |
| 16 | 0 | 3 | 0 | 0 | 2 | 0 | 0 | 10 | 0 | 0 | 5 | 0 |
| 32 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 10 | 0 | 0 | 3 | 0 |
| 64 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 2 | 0 |
| 128 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 |
| 256 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 |
| 512 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| Total | 0 | 38 | 0 | 0 | 45 | 10 | 0 | 73 | 13 | 0 | 50 | 17 |

antigen-antibody reaction so that gross agglutination is prevented. Or, ionic or electric rearrangements may occur intracellularly so that agglutination, again, is prevented in the aggregation phase. Whatever the mechanism involved, however, it seems that glucosamine in the manner used does prevent Rhesus agglutination specifically.

Antigen-Antibody Reaction without Agglutination

In 1957, Moskowitz and Carb(51), while testing the effect of pre-treating red cells with formalin before testing them against antibody to the ABO system, noted a peculiar phenomenon. Although there was no agglutination between, for example, group A cells and anti-A serum, the authors found that anti-A serum could be eluted from the non-agglutinated red cells by heating, and that adsorption tests revealed that the antibody did, indeed, disappear when reacted with pre-treated cells. Moreover, in all cases where the antisera was noted to disappear from solution and to be eluted from red cells, reacting such cells with anti-human globulin (Coombs serum) resulted in negative--no agglutination--tests. The authors interpreted these

findings as showing that despite the fact that formalin appeared to inhibit erythrocyte agglutination by appropriate (and specific) antisera, this effect was exerted probably by an alteration of cellular surface membranes and not by an alteration of the antigen or antibody or their reaction.

Along similar lines, Abelson and Rawson(1), while reporting their fractionations of anti-D serum by chromatographic procedures, noted that at times Coombs serum would not only fail to enhance, but actually seemed to depress, titers of the anti-Rhesus reaction; but they pursued this fact no further. In addition, in a report on his discovery of the hemagglutination-inhibitory effect of glucosamine, Hobel (34) was aware of the fact that his erythrocytes became discolored when incubated with glucosamine and anti-D antiserum. He felt that this brown discoloration represented a reaction between glucosamine and the erythrocyte, but he did not consider that this reaction might indeed be altering the erythrocyte so that hemagglutination as an indicator system for an antigen-antibody reaction was prevented. All these

findings were reminiscent of the work of Murray(52) who tested the sedimentation rate of cells involved with Rh hapten and found globulin still on the cells, of the work of Prager(61) who found that the cells which were protected from hemagglutination by various phosphate containing compounds, tested Coombs positive, and of the work of Dodd(17) who found that "conditioned" D negative cells would also adsorb anti-D serum without hemagglutination. In view of these reports, it was felt worthwhile to perform a series of experiments testing 1) whether, despite apparent agglutination-inhibition, antibody was attached to the red cell surface, and 2) whether merely by interaction with glucosamine the erythrocyte surface was altered and could no longer reflect antigen-antibody reaction. This series of experiments included the treatment of protected cells with anti-globulin serum, the attempt to build a lattice network on the cells for eventually agglutinating them, and the testing of cells, protected against agglutination, in hypotonic saline, in acidic pH fluid, and with sedimentation rates.

Testing with Anti-Human Globulin

In all experiments the anti-human globulin reaction was carried out on any erythrocyte-anti-D serum-glucosamine mixture where no agglutination was noted. The general procedure was that in all test tubes where Race scoring was "0," the cells were washed in 0.85 per cent sodium chloride and then centrifuged in a bench model centrifuge at 1,000 RPM for one minute to collect them into a compact button. The saline liquid was then removed so as not to disturb the cells, and the cells were resuspended in fresh normal saline--this process being repeated twice more. Then, following removal of the supernatant saline, the remaining cells were resuspended in 0.1 ml saline, and 0.1 ml anti-human globulin (commercial preparation by Dade Laboratories) was added to the suspension. The mixture was incubated at 20°C for one to two minutes, then centrifuged at 1,000 RPM for one minute, shaken gently, and agglutination was read according to the Race scoring method. While no consistently reproducible Coombs' score was obtainable, certain facts are noteworthy. In no case did a Coombs serum agglutination occur in a

negative control; and, invariably, where technique was accurate such agglutination occurred up to a titer of 512 in the positive agglutination control. In general, the greater the concentration of glucosamine from one per cent to six per cent, the less the Coombs serum titer was increased from its pre-Coombs agglutination. In no case, however, did the Race score increase more than fourteen points. For glucosamine solutions above six per cent, no or only minimal increase in Coombs serum titer was noted. In the experiment comparing the concentration of glucosamine to its inhibition of the Rhesus reaction, anti-human globulin reactions were run, as described previously, on all tubes where no macroscopic agglutination was noted. The results are given in Table VI(B).

From this table two facts are obvious. The inhibition of the anti-D serum reaction with D positive cells is not an all or none affair, but rather a continuous line from no reaction to complete inhibition. While an exact molecular relationship between glucosamine and the anti-D serum cannot be determined on the basis of the rather gross methods used in this experiment, such a

reaction can be postulated. Another fact is that when it is used in sufficient concentration, glucosamine does indeed appear to prevent the attachment of anti-D serum to the erythrocyte stroma, although it is possible that it may merely cover up Rhesus antigen-antibody complexes, thus preventing cellular aggregation and hemagglutination. To rule out this phenomenon, the lattice complex experiment of Coombs was repeated.

## Multiple Layer Technique

In 1951, Coombs and others(13) reported their ability to precipitate bovine erythrocytes previously classified as inagglutinable by building up an anti-globulin-globulin lattice on the sensitized cells. Their rationale for this procedure was that agglutination as an indicator of an antigen-antibody reaction might be prevented if the antigenic receptor site was below a relative or absolute "free wall surface." By adding alternately anti-globulin and globulin, they felt they had built up the lattice network on the original sensitizing antibody and so had produced hemagglutinaticn.

This lattice test or multiple layer technique was applied to the cells which glucosamine had protected

from hemagglutination. The basic hemagglutination-inhibition procedure was carried out in the usual way, utilizing a ten per cent glucosamine/0.2 M phosphate buffer inhibitor, both saline and albumin anti-D, a two per cent suspension of D positive cells, and incubation times of thirty and sixty minutes. Positive and negative controls were run and were normal. Inhibition was complete for all levels of both anti-D antisera. Then, 0.1 ml of the D positive cells protected against three levels (1:4, 1:16, and 1:64) of both complete and incomplete anti-D sera (which gave a titer of 1:128 in the initial testing manner) were washed in normal saline three times. They were then treated with 0.05 ml of anti-human globulin (see Coombs testing) for fifteen minutes at 20°C, washed again in normal saline, treated with 0.05 ml of non-specific human gamma globulin for fifteen minutes at 20°C--and the whole cycle repeated up to eight times. A negative control was run in the same manner utilizing the Rh positive cells which had been protected against twenty-two per cent bovine albumin. Other controls included erythrocytes which had been heated only and albumin in the place of human gamma

globulin or anti-human globulin to rule out non-agglutination. A summary protocol as well as the results of this experiment is given in Table VIII. In view of the fact that no agglutination occurred, it would appear that there is, indeed, no anti-D globulin attached to the erythrocytes protected by ten per cent glucosamine.

Three non-immunological tests were performed to ascertain whether the cellular membrane of the red cell had been altered following exposure to glucosamine; this, in turn, affecting the aggregation phase and preventing hemagglutination.

Sedimentation Rates

According to Jones and others(42), all other factors remaining constant, the sedimentation rate reflects the state of the erythrocyte surface. Sedimentation rates were carried out on 1) erythrocytes protected by ten per cent glucosamine and its dilutions from saline anti-D serum (1:4, 1:16, and 1:64) in the usual manner, 2) erythrocytes protected against albumin by glucosamine, 3) normal red cells heated to 37°C for sixty minutes, and 4) D positive cells reacted with incomplete anti-D serum in a saline media. Portions of

## TABLE VIII
## Results of Multiple Layer Experiment

Summary Protocol

Tube A: D pos cells protected with 10% glucosamine against anti-D serum (1:4)
Tube B: D pos cells protected with 10% glucosamine against anti-D serum (1:16)
Tube C: D pos cells protected with 10% glucosamine against anti-D serum (1:64)
Tube D: D pos cells protected with 10% glucosamine against anti-D serum (1:16)
Tube E: D pos cells, protected with 10% glucosamine against anti-D serum (1:16)
Tube F: D pos cells protected with 10% glucosamine against albumin (22% bovine albumin diluted 1:4)
Tube G: Untreated (heated only) D pos cells

Reagents applied to various tubes (sequence of events repeated five times)

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1) | ag | ag | ag | alb | gg | ag | ag |
| 2) | Wash all cells carefully | | | | | | |
| 3) | gg | gg | gg | gg | ag | gg | gg |
| 4) | Wash all cells carefully | | | | | | |

ag = antihuman globulin
gg = gamma globulin
alb= albumin

Results of adding antihuman globulin

| Addition | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

X=agglutination
0=no agglutination

all cells used in the sedimentation rate tests were first tested with Coombs serum, and only those cells treated with incomplete anti-D were found to be positive. The suspending media utilized in these experiments were 0.85 per cent sodium chloride, six per cent Dextran (of high molecular weight) in normal saline, and twenty-two per cent bovine albumin. Following pre-treatment, the erythrocytes were washed, made up to a twenty per cent suspension in the various media, dispensed into Wintroub sedimentation-hematocrit tubes, and allowed to settle at room temperature. Sedimentation was recorded in millimeters at intervals between thirty minutes and two hours, and up to twenty-four hours when an "overnight hematocrit" provided a control for equal suspension of cells.

Although too few tests have been performed to date for statistical significance, the sedimentation rates on these cells, in general, have revealed considerable variation both among the various media and among the same cells in the same media on different days. There has been a tendency, however, toward more rapid sedimentation by normal cells (that is, cells

which were only heated) in all types of media, it being most rapid in saline, next in Dextran, and finally in albumin. Rh positive erythrocytes which had been sensitized to albumin anti-D were found to fall at a much slower rate than normal cells; and the comparison between these cells and normal cells was reminiscent of the slowing of sedimentation rate noted by Ceppellini and Landy(10) on erythrocytes which they had conditioned with viral polysaccharides. This slowing may have represented an increase in viscosity possibly secondary to increased numbers of substances (molecules)--either polysaccharides or protein--on the red cell surface. Very slow sedimentation rates were noted for erythrocytes protected from hemagglutination by glucosamine; however, the reaction was non-specific since even those cells protected only from albumin showed the same phenomenon. In addition, there seemed to be some correlation between the amount of discoloration found in these cells, the slowness of their sedimentation, and their tendency toward hemolysis as recognized by a brown supernatant in the Wintroub tube and a decreased "overnight hematocrit." Ceppellini and Landy had reported that

some of their bacterial polysaccharides, adsorbed onto erythrocytes, had held them suspended indefinitely, until lysis occurred. This does not seem to be the phenomenon involved here. Instead, it seems either that the glucosamine converts the aggregation phase of the antigen-antibody reaction to a hemolysis phase (if, for example, all complement had not been inactivated by heating the antiserum to 56°C for sixty minutes), or that glucosamine in the presence of some proteins alters the cellular membrane of the red cell, weakening it or loosening the stroma so the hemoglobin is released, the brown color of the cells representing "hematin." Or, it might be that by altering cellular membranes glucosamine depresses the aggregation phase of the antigen-antibody reaction, depresses the sedimentation rate of the cells, but increases the mechanical fragility. The end result for all these cases would be the same, and the precise reason for the findings are unknown at this time. The possibility that the brown discoloration of the cells produced by glucosamine and the hemagglutination-inhibition attending it might represent an alteration of electrical surface charges was tested by the acid agglutination technique.

Acid Agglutination

In his discussion of antigens, Boyd(5) points out the thought and suggestion of some experimenters that denatured hapten protein or altered hapten polysaccharide may still be able to react with its antibody, but the reaction for some reason does not result in the formation of a precipitate--or, in our case, an agglutinate. Since, however, such antigen-antibody reactions on a red cell surface would be expected to alter the cell surface charge, acid agglutination of erythrocytes protected from hemagglutination by anti-D serum (1:4, 1:16, and 1:64) in the usual manner was tested. Such red cells, along with red cells merely heated to 37°C for sixty minutes and D positive cells protected by glucosamine from albumin, were washed three times in normal saline, and finally made up into ten per cent suspensions in normal saline. To 0.1 ml of this red cell suspension was added 0.5 ml of a range of McIlvaine's buffers, varying by 0.2 pH units. McIlvaine's buffer consists of 0.1 M citric acid and 0.2 M disodium hydrogen phosphate, pH range of 2.2 to 8.0; and the buffer was diluted by one-half in normal saline for use.

The point or pH where agglutination began was read for each erythrocyte sample tested.

To date an insufficient number of tests of acid agglutination has been performed to afford reliability to the results. The trend has been for the cells protected against anti-D serum to agglutinate at 0.2 pH units higher than the normal, heated cells or the cells protected against albumin. Thus, there is some indication of surface charge alteration; and, if the charge is such that a greater number of like charges is exposed to the surface, then the change is in the right direction.

Erythrocyte Fragility in Hypoosmolal Solution

In 1952, Bongiovanni and Abelson(4) tested the red cell fragilites of infants with erythroblastosis fetalis and found that they were decreased to both hypotonic saline and mechanical stimulation. They sensitized the erythrocytes from normal infants with incomplete anti-D serum and found that the fragilities here, too, were decreased from those of their controls. Greenwalt and Triantaphyllopoulos(29), on the other hand, in 1955, using spectrophotometric techniques to measure hemolysis instead of the grossly visible effect

used by Bongiovanni and Abelson found that hypotonic saline fragility was generally decreased in newborns, but elevated in infants with hemolytic disease, and that this elevation was not related to spherocytosis. They noted no difference between normal newborn and erythroblastotic newborn mechanical fragilities. They also found no correlation between the degree of fragility and the severity of the hemolytic disease.

In order to evaluate the effect of glucosamine on surface membranes, saline fragility studies were performed on 1) D positive erythrocytes protected from hemagglutination by prior incubation of anti-D serum with glucosamine, 2) D positive cells similarly protected from albumin anti-D serum, 3) D positive cells incubated with glucosamine and albumin, 4) D positive cells reacted with albumin anti-D serum in a saline environment, and 5) normal control cells merely incubated. Following their respective treatments, the cells were washed, made up into twenty per cent suspensions; and then 0.4 ml of the suspension was added to 3.0 ml of various hypotonic sodium chloride solutions ranging from 0.85 per cent to 0.24 per cent. The cells were then

refrigerated overnight at 4$^{o}$C. The next day the cells were centrifuged down, and the colored centrifugate was read on a Bauch and Lomb Spectronic 20 Photometer at 575 microwavelengths. Controls on the photmeter were set up by having cells from the subject read in the clinical laboratory by medical technologists ignorant of our results. Such tests have been run three times to date.

While no specific numbers were reproducible at these times, a few interesting observations were made. The first was that while the gross hemolysis control showed beginning hemolysis at 0.48 per cent sodium chloride, absorbance values on the photometer for the apparently clear supernatant of 0.56 per cent sodium chloride indicated some hemolysis. This discrepancy may represent the source of the divergent views of Bongiovanni and Greenwalt. Secondly, not only were all cells which reacted with glucosamine darker brown color than normal, but they revealed immediate, pronounced hemolysis at 0.80 and 0.75 per cents sodium chloride which was complete at 0.48 per cent sodium chloride. The sensitized erythrocytes, as in the sedimentation rate

experiment, were between these two extremes. The same arguments presented in the discussion of the results of the sedimentation rates could be advanced with respect to this hemolysis. The one basic fault with all these proposed explanations is that they do not account for the fact that albumin plus glucosamine give essentially the same results and discoloration that does anti-D serum with glucosamine. Again, in view of the minimally increased fragility noted with incomplete anti-D in a saline enviroment, it may be that it is the additive effect of a protein plus glucosamine which brings about the rapid discoloration and hemolysis of the erythrocytes while the agglutination reaction is prevented.

## DISCUSSION AND CONCLUSIONS

Since 1941, when the etiology and pathogenesis of erythroblastosis fetalis was elucidated by Landsteiner, Levine, and others, numerous methods have been proposed to prevent this disease. These methods include preventing translocation of antigen and antibody, destroying sensitizing antigenic fetal cells before they exert their effect, delivering infants pre-term before the maternal antibody exerts its effect. Another proposed method along this line of prevention has involved the neutralizing antibody or inactivating maternal titers prior to their transplacental passage. Various chemical agents--the "Rh hapten" of Carter, the ribonucleic acid derivitives of Hackel, the "unnatural sugars" of Boyd, and the hexosamine containing compounds of Cordova and Pirofsky--have been shown to inhibit the antibody *in vitro*; but none have been used sucessfully clinically. In 1964, Pearse and Hoble from our own laboratories reported the *in vitro* inhibition of the Rhesus antigen-antibody reaction by D-glucosamine-hydrochloride in ten per cent concentrations. As a sequel to their work this investigation had a two-fold purpose. The first

was to determine the effect of various physical and chemical factors on this inhibitory phenomenon; and the second was to elucidate the exact nature of mechanism of action of this inhibition.

The problem of the effect of osmolality on the inhibitory phenomenon of glucosamine was attacked in two ways. In one experiment it was shown that increasing concentrations (and so increasing osmolalities) of glucosamine produces increasing degrees of inhibition-- in so far as the Race scoring method can be quantitated. Because this effect was not associated with parallel increases in the osmolalities of the solutions tested, it appears that the inhibitory effect is unrelated to osmolality of the solution, _per se_, and that it reflects an action other than mere ionic hindrance or electron competition, although it may be either of these on a more specific order. The second experiment revealed that for given concentrations (osmolalities) of glucosamine solutions prepared with different diluents, the net effect is the same. These results indicate not only that the effect is independent of the diluent, but, again, that the effect is independent of the number of ions in

solution with glucosamine--depending, rather, on the amount of glucosamine.

The work on pH was consistent both with the fact that extremes of pH have been found to depr ss the associative phase of the Rhesus antigen-antibody reaction and with the fact that the inhibitory action of glucosamine is independent of pH as its mechanism of action. Also, comparing the Race scores of glucosamine with those of glucose show that the "inhibitory effects" of both these mechanisms are additive to a degree. In view of our brief work with acid agglutination of the glucosamine protected cells, it is interesting to note that this value is altered for these cells, and that it is altered in the direction expected--upward. This reflects the possibility that the surface charge of the cells has been altered by excess hydrogen or hydroxyl ions so that the charges are more nearly alike and tend to repel one another, thus repeling the cells from one another.

The universality or extent of glucosamine ibition was tested on the ABO and MN blood group systems in ten per cent concentrations, and its effect in preventing antigen-antibody reaction was negligible.

The similarity of this finding to that of other workers dealing with other agents has already been mentioned. We do not feel at this time that we can state, as they have stated for their agents, that the inhibitory effect is on the basis of the "inhibitor hapten" resembling the antigen and uniting with the antibody and inactivating it.

In view of the many instances which have been recorded of antigen-antibody reactions occurring without subsequent hemagglutination to indicate them, this possibility for glucosamine "inhibition" was investigated. The significance of this occurrence, of course, lies in the fact that the prevention of erythroblastosis fetalis is concerned not with the prevention of hemagglutination, per se, but rather with the prevention of the antigen-antibody reaction resulting in hemagglutination. In addition, we were clinically interested in glucosamine as an agent which would combine with antibody and prevent its subsequent reaction with antigen.

In the time sequence series of tests we had fairly well concluded that if the antigen-antibody reaction is allowed to occur, glucosamine will not inhibit it. Now, if glucosamine exerts its effect

by resembling a hapten, this result would be expected. If it acts strictly as a hapten, however, then one would think that mixing it with antibody for ten or thirty minutes would give the same result--and this was not the case. On the other hand, if glucosamine exerted its effect by changing cellular membranes, then it would be thought both to exert its effect primarily despite the occurrence of the antigen-antibody reaction and to exert the same effect whether it was incubated with antibody for ten or thirty minutes. This first objection could be overcome by supposing that glucosamine can alter surface membranes only if it acts before or simultaneously with antibody, but the second can not.

To clarify these problems Coombs reactions were carried out on all cell samples protected from hemagglutination. Up to initial glucosamine concentrations of six to seven per cent, occassionally positive Coombs tests occurred, indicating that there was antibody attached to the red cell surface. In many samples where this occurred, the anti-D serum was diluted only 1:2 or 1:4; and it is possible that other non-specific reactions had occurred. No positive Coombs tests were

noted when the concentration of glucosamine was nine or ten per cent. Nor was it possible to build up a globulin-antiglobulin lattice on such protected cells and so possibly promote or induce agglutination.

Initial studies to evaluate the effect on surface membrane structure of incubating red cells with glucosamine and anti-D serum have not yet been performed in sufficient quantities to permit definite conclusions, statistically valid. Further tests are now in progress. To date, however, certain trends are apparent. Such incubation procedures cause cells to have a decreased sedimentation rate, an increased fragility to hypoosmolal solutions of saline, and an elevated acid agglutination point. Since each of these parameters reflects on the state of the cell membrane, and since all have been altered, it seems valid to conclude that the cell membranes have been altered on exposure to glucosamine. To be sure, albumin, the mere heating of cells, and other controls also induce similar trends to the red cell; but none, nor any combination, is as effective as the glucosamine-anti-D serum one. It is now our opinion, based on the

tests indicating an absence of antibody from the erythrocytes and on tests indicating altered surface membranes, that glucosamine inhibits the Rhesus antigen-antibody reaction by preventing the attachment of the antibody to the antigen within the erythrocyte stroma, and that it prevents this attachment by altering the red cell stroma or surface.

To be sure, there are many other experiments which should be done to corroborate and extend this impression; and many of the tests for cellular membrane change must be repeated for true significance. However, it also seems obvious, in view of the technique, that the ribonucleic acid derivitives, the unnatural sugars, and the numerous hexosamine compounds which have been found to inhibit this same antigen-antibody reaction should be tested in a manner similar to ours before the nature of their inhibitory ability is accepted as it has been proposed.

## SUMMARY


1. The history of the elucidation of the etiology and the pathogenesis of erythroblastosis fetalis is presented.

2. The pathogenesis of hemolytic disease of the newborn is discussed, along with the numerous methods proposed to prevent its occurrence.

3. The Rhesus antigen, antibody, and antigen-antibody reaction is discussed.

4. The effect of pH, osmolality, concentration, and time on the ability of glucosamine to inhibit the Rhesus reaction was tested.

5. The universality of glucosamine inhibition was examined.

6. Experiments were performed to determine if antibody were present on non-agglutinated red cells or if glucosamine altered cellular membranes of the cells.

7. Results of the experiments were discussed.

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In his dialogue, the *Republic*, Plato comments on the divine national state, stating that because it must exist, it can exist and can be built without the aid of some individual members. However, he goes on to say, when all individual members contribute to the state and do their share, then it exists in greater splendor and in turn yields greater pleasure and comfort to its members. In an analogous sense, this required thesis would have been accomplished had there not existed some of the persons who helped in its preparation. The work was there to be done and it would have been done. But because these people were around--and helpful, and contributing, and aiding--this thesis was accomplished more easily, more pleasurably, more scientifically, and, I hope, more profitably.

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