SEROLOGICAL RELATIONSHIPS BETWEEN THE Rh ANTIGENS OF THE D-D^u SERIES

by

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INTRODUCTION

Fifty-five years have elapsed since it was first realized that human erythrocytes may show inheritable intra-species differences distinguishable by means of appropriate serological tests. Within a short time sufficient evidence accumulated to make possible the division of the human race into four distinct blood groups. Only sporadic advances took place in the next four decades, but beginning with the discovery of the Rh factors as an impetus, numerous human blood groups have been identified. While the term "blood groups" is often used as a generality in referring to the original A-B-O types, it is more correct to refer to "systems" of blood groups. Each blood group system contains factors which are apparently serologically distinct, but related genetically. In all of these systems, the only means for distinguishing the entities within them is by use of serological techniques. No chemical or physical tests of sufficient delicacy for analyzing the differences have been devised.

While antigenic differences are disclosed by serological methods, the relationships of the antigens within a blood group system are demonstrable almost entirely from genetical data. As far as the gross serological aspects are concerned, there is no more resemblance between antigens A and B of the classical system of human blood groups than there is between pneumococcus polysaccharide and the capsular material of Friedländer's bacillus, but the mode of inheritance of A and B indicates their relationship. This introduces a complexity in interpreting the data derived from experimental studies of human erythrocyte antigens, since the evidence

must be considered simultaneously from the serological and genetical points of view. The extent of our knowledge in either field is limited, so there is ample opportunity for controversy to arise as contrary interpretations and wide generalizations are derived from the same experimental observations.

Experiments have shown that a simple hapten with only one reactive group may induce the formation of a multiplicity of antibodies, which may in turn cross-react with a variety of antigens other than the one bringing about their formation. When an antigen is more complex and has a comparatively unknown chemical structure, as in the case of blood group antigens, the interpretation of the serological picture is exceedingly difficult.

Part of this complexity is reflected in the usual course of events following the description of a new blood group system. First, one or more distinct antigens are recognized by means of apparently highly specific antibodies. Further investigation then discloses "subgroups" which are usually distinguished by the weak or irregular reactions given in the presence of otherwise satisfactory typing serums. These variants are not simply defective cells produced by metabolic errors in occasional individuals, but are actually different antigenically, as shown by their hereditary nature. Any antigen within a system may show this subtype phenomenon. Attempts to explain the nature of the differences between the "regular" antigen characteristic of a blood group factor and its intermediate variants have been hampered by gaps in serological and immunological knowledge. Among the factors which make the data difficult to analyze are: (1) lack of information concerning what constitutes an antigen; (2) inability to predict how the antibody-forming mechanism will react when confronted with

an antigenic substance; and (3) poor understanding of the dynamics of in vitro antigen-antibody reactions. In an effort to obtain information aimed at clarifying matters such as these, the investigations reported here deal with the D^u subgroup of the D antigen of the Rh-Hr blood group system.

This study is divisible into three parts: (1) the determination of the incidence of type D^u bloods in Utah; (2) the development of a classification for D^u bloods on a more rational basis than has been done previously; and (3) the more precise definition of the similarities and differences between D and the D^u spectrum by means of absorption, elution and cross-reaction techniques.

REVIEW OF THE LITERATURE

Numerous experiments dealing with serological inter-species specificity were conducted in the decade 1890-1900 (Ehrlich, 1910). This apparently induced Landsteiner to investigate the possibility of intra-species serological differences. His first attempts involved the mixing of serum from one human with the erythrocytes of another and vice versa. Admittedly, he expected to find that agglutination or hemolysis, if they took place at all, would be of a minor nature. In many of the mixtures, however, prompt and very marked reactions took place. Landsteiner's (1900) first paper concerning this effect was only a brief description of what actually occurred. A year later Landsteiner (1901) was able to state that humans could be divided into three distinct blood groups on the basis of their serological activity. He assumed that this distinction was due to two antigens, the red cells of some individuals having one of the antigens, those of other persons having the second, while the erythrocytes of still other humans deviated by having neither antigen. It was pointed out that if either or both of the antigens were missing, the serum would contain antibodies specific for the absent components. This dictum has come to be known as "Landsteiner's Rule". A fourth group was discovered by von Decastello and Sturli (1902). This did not disturb the two-antigen hypothesis since red blood cells of the new group acted as though they contained both antigens previously described, while the serum contained no antibodies. These groups are now known as O, A, B and AB.

von Dungern and Hirszfeld (1911) produced evidence to show that a system of only four blood types was an oversimplification. If anti-A serums were absorbed by certain (not all) type A cells until the serums no longer

reacted with the absorbing blood, an antibody still remained which would agglutinate the majority of bloods of group A and AB. This led them to postulate the existence of two kinds of A and AB blood. The experimental results were confirmed by Schütze (1921), who made no attempt to explain them. Guthrie and Huck (1923) and Coca and Klein (1923) made similar observations and came to identical conclusions, i.e., the results were due to a third antigen and its corresponding antibody (designated cC by Guthrie and Huck, Xx by Coca and Klein). Both groups suggested that Landsteiner's scheme should be modified accordingly. Lattes and Cavazzuti* (1924) held that these subtype differences, since they were confined entirely to blood of A (or AB), did not represent a new group but was simply a manifestation of the quantity of A antigen present in the erythcytes. Absorption experiments proving that the weakly reacting cells could remove all of the anti-A antibody from a serum appeared to substantiate their belief. Observations made by Landsteiner and Witt (1926) and by Landsteiner and Levine (1926) were interpreted by these authors as indicative of qualitative differences between the A subgroups. Especially convincing was the finding that serums from A_2 or A_2B individuals often contained anti-A_1 agglutinin and more rarely, serums from A_1 persons might react with A_2 cells. (Landsteiner's paper with Levine made what is probably the first mention that anti-A_2 antibodies cross-react with type O cells.) Elution experiments augmented their conviction, for when anti-A

*In the light of the present controversy concerning Rh nomenclature, it is amusing to note the complaint in this article directed against Guthrie and Huck, who utilized lower case letters to indicate antigens and capitals for antibodies. They were accused of creating deplorable confusion since standard usage was exactly the reverse.

agglutinins were split off from A cells, two kinds of antibody were found, one acting on A_1 and A_2, the other on A_1 red cells only.

Further subdivisions have been made with regard to A, including A_3 recognized by Fischer and Hahn (1935) and Friedenreich (1936a), A_4 by Gammelgaard and Marcussen (1940), and A_5 by Hirszfeld and Amzel (1940). A very peculiar A variant, A_0, was described by Grove-Rasmussen and Levine (1952). Erythrocytes of this subtype were agglutinated only by serums from type O persons, no reaction being obtained when the anti-A serum was derived from group B. Immunization of rabbits with A_0 resulted in the production of the usual anti-A serum. Antibody eluted from A_0 cells appeared to contain large amounts of the substance acting on the component A common to A_1 and A_2 cells.

Landsteiner and Levine (1926) and Witebsky (1948) noted transitional forms between A_1 and A_2, indicating a graded series. Witebsky would reclassify into this transitional group about 60% of the bloods which have been typed as A_2 with absorbed B commercial serums.

The A subgroups are of so little practical importance that they are generally disregarded in pretransfusion typing, although Davidsohn (1937) and Wiener (1941a) felt that intragroup differences (especially if the recipient has previously formed A_1 or A_2 agglutinins) may account for the lack of success in inducing the expected rise in the hemoglobin level of the recipient, even though overt symptoms of transfusion reaction may be absent. Such cases, however, appear to be very rare, judging from the paucity of reports concerning them.

Numerous unsuccessful attempts have been made to demonstrate subtypes of B by absorbing various group A serums with B erythrocytes and testing

the absorbed serums against other B bloods (Wiener, 1943c). When only human reagents were used, homogeneity in group seemed to be the rule. If animal red cells were used for absorption of human anti-B serums, it appeared that type B may be divisible. Friedenreich and With (1933) found that some human anti-B serums could be absorbed completely with small quantities of rabbit cells, whereas even large amounts failed to absorb the antibody from others. Chicken red cells might also achieve either partial or complete absorption, depending on the serum, and would sometimes remove antibody left after rabbit absorption. The explanation given for this phenomenon was that all human erythrocytes have a component in common, B_i; in addition, other factors, B_{ii}, B_{iii}, etc., can be present. Human serums may contain antibody against these structures in any combination. Rabbit and chicken cells appeared to possess certain of these component structures, but not the same ones. Hence, they may or may not remove all anti-B agglutinins, depending on the qualitative antibody content of any given serum.

The M-N system of Landsteiner and Levine (1927a, 1927b, 1928) has also been shown to contain subgroups. M variants were demonstrated by Friedenreich and Lauridsen (1938) and Jakobowicz, Bryce and Simmons (1949). Crome (1935), Friedenreich (1936b), and Pietrusky (1940) described an N_2 subgroup. The latter group occurs very rarely; it has been found only eight times in 20,000 paternity cases (Andresen, 1947). Variations of M have been reported even less frequently. Wiener (1938) felt that he had detected five M and two N components by means of anti-M and anti-N serums tested against the erythrocytes of apes and various species of monkeys.

A more recent development in connection with the M-N system was the description of an antiserum reacting with a previously unknown blood factor,

S (Walsh and Montgomery, 1947). Detailed studies of this anti-S antibody by Sanger and Race (1947) made it apparent that the serum was reacting with an antigen associated genetically with M and N, either in close linkage or as part of a multiple allele system. Pedigree studies and statistical considerations have verified the findings. Levine, Kuhmichael, Wigod and Koch (1951) identified still another new factor and presented evidence to indicate its allelomorphism with S. The logical designation, s, was given to this antigen.

The MNSs series does not have antibodies corresponding to Landsteiner's Rule. As a matter of fact, M and N are notoriously poor antigens in man and typing serums for them are usually prepared in rabbits. Altogether, there have been less than thirty cases in which anti-M or anti-N antibody has been found in human serums (Race and Sanger, 1950). In most of them, the antibody seems to have been the result of immunization. S may be somewhat more antigenic, but neither S nor s has been studied sufficiently to draw any firm conclusions.

The P factor (Landsteiner and Levine, 1927b, 1928) was discovered in the same series of experiments that disclosed the M and N antigens. The classification of humans on the basis of this antigen is simply that of P-positive or P-negative, the allelomorphic antigen, p, never having been found. In their first mention of P, Landsteiner and Levine noted that there were at least two classes of P-positive blood, strongly and weakly reacting. The work of Henningsen (1949) indicates that P is expressed as a graded series of reactions, not divisible into distinct subgroups on the basis of strength of reaction. Using a highly sensitive technique, Henningsen showed that the serum of P-negative persons contains

anti-P with a regularity close to that of the A-B-O isoagglutinins.

Landsteiner and Wiener (1937) reported an antibody with anti-M characteristics formed in guinea pigs which had been injected with erythrocytes from Rhesus monkeys. Continued work along the same lines, aimed primarily at investigating inter-species M relationships, resulted instead in the discovery of a new antigen, Rh, in the Rhesus red cells (Landsteiner and Wiener, 1940, 1941). Antiserums produced in the guinea pig against this property were capable of agglutinating Rhesus erythrocytes, and when absorbed free of species specific agglutinins, could clump the erythrocytes of about 85% of Caucasians. The antigen itself they termed the Rh factor. Humans whose erythrocytes were agglutinated by the guinea pig antiserums were called Rh-positive, the remainder, Rh-negative. Wiener and Peters (1940) studied the serums from three patients who had suffered reactions after multiple transfusions despite donor-patient compatibility with regard to the A-B-O types. In each of them an antibody was found which corresponded to the Landsteiner-Wiener anti-Rhesus type. Additional evidence incriminating the Rh factor was that the patients were Rh-negative while the particular donors whose blood had caused the transfusion reaction were Rh-positive. This was by no means the first time that atypical agglutinins had been observed in patients who had received multiple transfusions, for Unger (1921), DeGowin and Baldridge (1934), Neter (1936), Zacho (1936), and Culbertson and Ratcliffe (1936) reported such instances. However, the Rh factor was unique in being definitely characterized and named.

In the previously-mentioned paper, Wiener and Peters suggested that a case described by Levine and Stetson (1939) might have its origin in Rh

incompatibility. This paper described a severe reaction which took place on the first transfusion of A-B-O compatible blood to a postpartum woman. An atypical warm agglutinin reacting with about 80% of random bloods was found in her serum. The authors theorized that the original stimulus to antibody production was a dominant hereditary factor present in the fetal blood, but absent from the maternal circulation. Since the patient had already developed antibody through pregnancy-induced sensitization, a serious reaction occurred when she was transfused for the first time with blood containing the same antigen as that of the fetus. In 1940 the case was reviewed and the findings were as expected--the mother was Rh-negative, father Rh-positive, and donor of the reaction-causing blood, Rh-positive. A succession of investigations (Levine and Katzin, 1940; Levine, Katzin and Burnham, 1941) of other occurrences involving reactions to first transfusions of apparently compatible blood revealed some salient facts: (1) pregnant or recently delivered women were involved; (2) all were Rh-negative; (3) the incidence of fetal and neonatal morbidity in the infants of these mothers was abnormally high; and (4) when adequately done, clinical and pathological examination established the disease of the infants to be erythroblastosis fetalis. By themselves, these data were quite convincing, and in a short time more evidence was available to establish beyond any question the role of the Rh factor in hemolytic disease of the newborn (Levine, Vogel, Katzin and Burnham, 1941; Wiener, 1942; Burnham, 1941; Javert, 1942; Potter, Davidsohn and Crunden, 1943; Brown and Levine, 1943). All of this vindicated the shrewd guesses of earlier workers, as Dienst (1905) had mentioned the possibility of transplacental immunization from fetal blood, while Parsons, Hawksley and Gittens (1933) as well as

Darrow (1938) had given consideration to the fact that erythroblastosis was a transplacental hemolytic disease.

The serums found in the beginning were all alike in giving approximately 85% positive reactions and in having the same specificity as the anti-Rhesus guinea pig serums. Others were soon described, one giving 87% positives (Levine, Burnham, Katzin and Vogel, 1941), another 70% (Wiener, 1941b) and still another only 30% positive tests (Race, Taylor, Boorman and Dodd, 1943; Wiener, 1943a).*

At the time that the three serums, 87%, 85%, and 70%, were known, the situation appeared to be analagous to that of the subgroups of A, but it quickly became clear that the Rh types were more complicated and would not parallel the A subgroups. Thus, occasional bloods were encountered which failed to react with 85% serums, but were agglutinated by the 70% and 87% varieties (Landsteiner and Wiener, 1941). After the discovery of the 30% positive serum, it was realized that each serum studied contained a single Rh antibody specific for one antigen with the exception of the 87% kind which apparently contained a mixture of 85% and 70% antibodies (Wiener, 1943a; Davidsohn and Toharsky, 1942; Levine, 1942b, 1943b). On the basis of the various types of serums and the antigens disclosed by them, Wiener and co-workers proposed several systems of nomenclature before deciding on one which has been maintained to the present time in relatively unmodified form (Wiener, 1943a, 1943b, 1943c, 1944a, 1945a, 1946c, 1950; Wiener and Sonn, 1943; Wiener, Sonn and Belkin, 1943; Wiener and Landsteiner, 1943; Wiener, Sonn and Polivka, 1946).

*To convert the various systems of nomenclature and percentage reactions to present terminology, see Table 1.

Table 1

PRESENT Rh NOMENCLATURE

SERUMS			ANTIGENS	
% DESIGNATION OF SERUMS	PRESENT NOMENCLATURE		FISHER	WIENER
	FISHER	WIENER		
87	Anti-C ≠ D	Anti-Rh_1	C	rh'
85	Anti-D	Anti-Rh_o	D	Rh_o
70	Anti-C	Anti-rh'	E	rh"
30	Anti-E	Anti-rh"	C^w	rh^w
			D^u	Germanic-Rh_o

Rh TYPES		Rh SUBGROUP TYPES	
FISHER	WIENER	FISHER	WIENER
C	rh'	C^w, C^u	rh^w
D	Rh_o	D^u	Germanic-Rh_o
E	rh"	E^u	
CD	Rh_1	CD^u	Germanic-Rh_1
DE	Rh_2	D^uE	Germanic-Rh_2
CDE	Rh_z	CD^uE	Germanic-Rh_z
CE	rh_y	C^wE, C^uE, CE^u	rh^w_y
cde	rh	c^vde	

Yet another factor was found by Levine (1941) and Levine, Burnham, Katzin and Vogel (1941) who realized that the new antigen and antibody were related to the Rh system, but acted in a fashion antithetical to Rh serums, agglutinating almost all Rh-negative erythrocytes and many of the Rh-positive ones as well. The reverse nature suggested use of the term Hr. The antibody in Levine's serum was weakly reacting, preventing him from drawing firm conclusions. A stronger serum of the same type found by Race and Taylor (1943) definitely established the reciprocal nature between Hr and Rh and showed that the reciprocity was between the Hr factor and the 70% Rh factor only. Fisher (1944), using this contribution to reason that there must be two more Hr antigens remaining to be discovered, proposed the CDEcde system of nomenclature to include them. (See Table 1.) The anti-e predicted by Fisher has been found (Mourant, 1945) and anti-d serums have been identified by Hill and Haberman (1948) and Hill, Haberman, Guy and Davenport (1950), thus fulfilling Fisher's hypothesis.

Before and during the developments mentioned in the preceding paragraphs, the realization had been growing that the complete story of Rh and erythroblastosis was still not known. No Rh or other atypical antibody could be found in the serums of about one-half of the women who gave birth to erythroblastotic infants, and in many of the cases in which antibody was found, the <u>in vitro</u> reaction was so weak that it hardly seemed conceivable that so much damage could be caused in the infant (Levine, 1942a, 1943a; Diamond and Abelson, 1945). If erythroblastosis were a syndrome having two or more causative agents, one of them being the Rh factor and the others unknown, this could be explained easily. The fact

that the mothers were almost invariably Rh-negative and the involved infants and their fathers, Rh-positive, appeared to neutralize this argument. The second obvious possibility was that a kind of antibody not demonstrable by traditional saline tests was responsible. Wiener (1944c) and Race (1944) independently furnished the proof that the latter explanation was correct when they developed an agglutination inhibition test. Essentially the procedure involved incubating a saline suspension of Rh-positive cells with the unknown serum, washing the erythrocytes, and adding a drop of known saline-active D serum. If the reagent agglutinated the cells, the test was negative. Failure to secure agglutination indicated that something in the unknown serum had combined with the antigen and blocked the action of a serum that ordinarily would have produced good agglutination. This phenomenon led to the use of the term "blocking antibody". The method was very insensitive, but did prove the important point that an antibody existed which was incapable of agglutinating suitable erythrocytes, although it could combine with the antigen they carried. Diamond and Abelson (1945) produced the first evidence that this blocking antibody could be made to act as an agglutinin. When they mixed a drop of whole blood (approximately 40% cell suspension) with a drop of anti-Rh serum containing blocking antibody on a warmed glass slide, massive agglutination occurred. Wiener (1945b) extended these observations further, showing that the heavy cell suspension alone was not the answer, but that the reduction in saline concentration was essential. A 2% suspension of Rh-positive cells could be agglutinated by blocking antibody provided that serum or plasma, rather than physiological saline, were used as the suspending media. Other substances later found to be useful in promoting

the action of this kind of antibody were albumin (Diamond and Denton, 1945), gum acacia and polyvinyl alcohol (Levine, 1946), gelatin (Diamond and Denton, 1945; Fisk and McGee, 1947; Prokop, 1951), dextran (Grubb, 1949; Jones, 1950) and polyvinylpyrollidone (Formaggio, 1950; Goudemand and Samaille, 1950; McNeil and Trentelman, 1952a, 1952b).

The blocking technique devised by Wiener and Race indicated that the effect of saline was inhibitory rather than destructive. This was confirmed more directly by Diamond and Denton (1945) who incubated blocking serum and Rh-positive cells suspended in saline, washed the cells, and resuspended them in bovine albumin, after which agglutination took place readily.

Wiener (1944c) has postulated that the molecule of the saline antibody is "bivalent", possessing two or more reactive groups capable of combining with different red cells and so agglutinating them. The blocking type was supposed to be "univalent" with only one combining group. He explains the action of protein materials in activating the antibody by his "conglutination" theory (Wiener, 1945b, 1946a). Briefly stated, a hypothetical "X-protein" or "conglutinin" present in serum, plasma, etc., acts as a bridge through which the univalent antibody can function. This represents the only theory advanced to date dealing with the reaction peculiar to the blocking antibody. However, evidence exists that this theory is untenable (Argall, 1951).

Univalent antibodies are found more frequently in the serums of mothers of erythroblastotic infants than are the bivalent ones (Wiener, 1946b). Wiener (1946a, 1946d) explains this by assuming that their small size allows greater placental permeability, permitting increased concentration

leading to the most severe forms of hemolytic disease.

In addition to the methods already discussed, the Coombs anti-human-globulin technique (Coombs, Mourant and Race, 1945) and the proteolytic enzyme test (Morton and Pickles, 1947) were introduced. Both procedures permit the detection of blocking antibody in the presence of sodium chloride. They apparently differ from each other in mode of action, and neither bears any obvious serological relationship to methods employing colloidal media.

A number of suggestions have been made for giving names to the saline-inactive antibody. Some were based on the supposed role of the antibody clinically, some on its postulated mode of action, others on the type of suspension medium employed for the red cells. However, until a more logical basis for nomenclature and more experimental evidence are available, it seems better to use the commonly recognized term "blocking antibody" to depict all forms of saline-inhibited antibody, with the understanding that the designation is possibly too comprehensive.

The stimulus provided by the discovery of the Rh factors and application of the methodology developed for the detection of blocking antibody has resulted in the discovery of many new blood group systems in man. In studying antigens within the Rh system, it is highly important to be aware of these other types since supposedly pure Rh serums may contain antibodies against other factors (Rosenfield, Vogel, Miller and Haber, 1951).

Subgroups had been found in every human blood group system studied thoroughly, so it was not surprising that a similar situation should be found with regard to the Rh system. Wiener (1944b) was the first to note the existence of "intermediate" Rh types which gave weaker reactions with

standard typing serums than did the variety of Rh-positive bloods most frequently encountered. The phenomenon was consistent in any individual and, from its familial incidence, appeared to be inheritable. This first observation of an Rh subgroup was in connection with the antigen D. No attempt was made to characterize the subgroups although Wiener, Sonn and Belkin (1944) mentioned that they might be the cause of certain minor, but persistent, variations from the six-gene multiple allele theory.

Subgroups for each of the Rh antigens have now been described. With one exception, they are similar in their serological action; i.e., no antiserum has been found which is specific for bloods of the subgroup, as distinguished from those of the "regular" kind found in the vast majority of individuals of a particular group. The lone example of a specific subgroup antiserum is the anti-C^W first found by Callender and Race (1946) and subsequently by a number of investigators. From its description, it is obviously a variant of C. The patient in whom the antibody was originally identified was a homozygous C, but persons of type Cc and cc can also form anti-C^W (Lawler and van Loghem, 1947; van Loghem, Bartels and Hart, 1949). While C^W antiserums are highly specific, agglutinating only C^W cells, anti-C serums may be less restricted. Callender and Race (1946) found that the majority of anti-C serums were actually anti-C $\neq$ C^W, only a few being pure C. In absorption experiments, Race, Sanger and Lawler (1948c) were able to remove all of the antibody from anti-C $\neq$ C^W serums by absorption with either kind of blood, C or C^W. With pure C antiserum or artificial mixtures of pure C and pure C^W, only the homologous cells were active as absorbing reagents.

Other variants of C are C^U and c^V. Race, Sanger and Lawler (1948a)

noted that when they tested 284 random samples of blood with anti-D, E, e, c and C^w serums, plus three different anti-C serums, two samples gave irregular reactions with the anti-C serums. The irregularity was not parallel so it appeared that while both were subgroups of C, they were not identical. No further studies were made with the one labelled C^u, but the other (c^v) gave some very interesting reactions. Blood of the type c^vDE was found to give a "double-dose effect" when titrated with three anti-c serums.*

In genetic studies, c^v appears to be more nearly related to c than to C. Absorption of anti-C $\neq$ c^v serum with c^v cells is as effective in removing all antibody as is absorption with C cells. Whether or not c^v erythrocytes can also remove anti-c antibody as well was not mentioned. A second example of c^v was found by Jones, Steinberg, Allen and Diamond (1953). These authors confirmed the dosage effect with anti-c and furnished additional presumptive evidence for the relationship to C. This was based on the observation of a new antibody, anti-f, described by Rosenfield et al. (1953). The antigen defined appears only in individuals with at least one chromosome containing c and e. For example, Cde is f-negative while cDe or cde are f-positive. Jones et al. (1953) were able to obtain blood from two individuals with a genotype CDe/c^vDe and found both to be f-negative. This indicates a C-like characteristic. c^v, then, appears to be an unique intermediate--a partial antigen

*It has been found that the Hr serums anti-c and anti-e will show much higher titration values when tested against cc or ee cells respectively than when tested with Cc or Ee. This is the "double dose effect" (Race, Taylor, Boorman and Dodd, 1943). Landsteiner and Levine (1927) noted a similar effect with M and N serums.

representing the simultaneous product of two alleles, C and c, which, by the ordinary rules of genetical theory, must be on separate chromosomes.

One example of an E subgroup, E^u, was reported by Armytage, Ceppellini, Ikin and Mourant (1950), a second by Mourant et al. (1952). These were recognized by weak and irregular reactions with anti-E serums.

The established clinical importance of the D antigen should merit focussing special attention on its subgroups, although serologically and genetically they are of no greater significance than the C or E intermediate types. After the first brief mention by Wiener (1944b), little attention appeared to be given the D subtypes until Stratton (1946) published a case which afforded a better opportunity to demonstrate the hereditary pattern. He proposed the term D^u in accordance with the British system. Wiener (1950) suggested Germanic-Rh_o, Germanic-Rh_1, etc.* The particular D^u type in the family studied by Stratton was recognized by irregular reactions with anti-D serums of the saline kind. The erythrocytes were agglutinated by some serums, but gave negative reactions with others, despite the fact that all of the serums were approximately equivalent when tested against D red cells. Serums of the first kind he considered to be anti-D $\neq$ D^u, the latter pure anti-D. Absorption with D cells was found to take out all of the antibody. Race, Sanger and Lawler (1948b, 1948d) made several observations which can conveniently be summarized as follows:

(1) D^u red cells are agglutinated by some saline anti-D serums,

*Wiener (1949) scolded Stratton for introducing a term with no meaning, stating that only factors for which there was a specific antiserum (e.g., C^W) should be given recognized designations, but a year later introduced these symbols.

but not by others. Eluates prepared from cells which react, as well as cells giving no visible agglutination with a particular serum, show saline anti-D activity. Cells treated with blocking anti-D yield blocking anti-D upon elution.

(2) D^u cells are usually agglutinated in albumin by saline anti-D.

(3) D^u cells are sensitized for the anti-globulin test by the great majority of blocking anti-D serums.

(4) D^u cells are usually not agglutinated in albumin by incomplete anti-D.

(5) D^u cells are efficient in removing anti-D agglutinin from a saline anti-D serum which agglutinates the cells concerned. Some D^u cells, although not reacting visibly with a given D $\not$ D^u saline serum, can nevertheless reduce the anti-D titer.

Also mentioned was the peculiarity of some cells showing anti-D antibody in the eluate, although causing no detectable decrease in titer of a serum.

Stratton and Renton (1948) reached almost identical conclusions independently. In addition, they made the statement that it is very improbable that even the lowest grades of D^u can make anti-D antibody.

Stratton and Renton (1949) set up a rather casual classification of D^u using the terms "high grade" and "low grade" based on the reaction of erythrocytes with a battery of serums. Those that were agglutinated by 0-45% of saline anti-D $\not$ D^u serums were high grade; those reacting with none, low grade. The following table taken from this paper indicates the type of reaction to be expected.

Table Showing Percentage of Anti-D Sera Giving Positive Results (the Actual Percentage of Positive Results Depends on the Individual Cell Sample Tested)

Test	Antigen in Cells			
	D	High-grade D^u	Low-grade D^u	d
Agglutination in albumin*.	60-90%	0-10%	Nil	Nil
Agglutination in saline'..	60-100%	5-45%	Nil	Nil
Coomb's reaction*.....	90-100%	70-100%	20-60%	Nil

*The sera tested were the strongest available.
'The sera tested were a random selection.

In the same paper additional evidence was introduced that D^u persons could not produce anti-D antibody. A definite statement was made that D^u should be considered Rh-positive for reporting the Rh type of prenatal patients.

The incidence of D^u varies from race to race. It is high in Negroes (Wiener, Unger and Sonn, 1945) and in persons of Eastern Mediterranean stock (Rosenfield, Vogel, Miller and Haber, 1951; Choremis et al., 1953). Renton (1950) and Rosenfield, Vogel, Miller and Haber (1951) have made the only studies of Caucasian populations and their results are markedly different.

In addition to being of interest for their in vitro serological properties, at least two Rh subgroups are of some clinical importance themselves. C^w has been associated with transfusion reactions (Callender and Race, 1946; Collins, Sanger, Allen and Race, 1950) and erythroblastosis

(van Loghem, Bartels and Hart, 1949). D^u cells were responsible for a transfusion accident when a dd patient who had already produced anti-D antibody was given supposedly D-negative blood which on re-examination was actually found to be D^u (Diamond and Allen, 1949). The antigenicity of the intermediate Rh type was demonstrated by van Loghem (1947). Attempting to induce formation of anti-C by injecting Cde erythrocytes into a ccdd volunteer, he found both anti-C and anti-D in the serum. The donor whose cells were used was found to have the Rh type CD^ue. Mollison (1949), Rosenfield, Vogel, Miller and Haber (1951), and Wiener and Brancato (1952) described cases of erythroblastosis in which the affected infant and its father were D^u, while the mother was dd.

D^u is novel in one respect among Rh subgroups. It is the only one in which a subgroup individual has formed antibody apparently directed against the main antigen of the group. Argall, Ball and Trentelman (1953) reported the case of a woman whose erythrocytes gave all of the typical reactions for D^u, but whose serum contained a high titer, 1:128, of antibody conforming specifically to anti-D.

In the most comprehensive study of the D^u subgroup made to date, Race, Sanger and Lawler (1948b) stated that their report was only of a preliminary nature and that "it is clear that there is much yet to be learnt about D^u, and we feel that the subject is capable of affording fundamental knowledge, both serological and genetical".

MATERIALS AND METHODS

PART I
Detailed Techniques

The methods employed are outlined in the following paragraphs. They are referred to thereafter by the name given in the paragraph heading.

A. Saline tube test:

(1) A 2% suspension of erythrocytes was made in 0.85% sodium chloride solution, washed, and restored to volume in the same medium.

(2) 0.05 ml of serum and the same amount of antigen were placed in a 10 x 75 mm test tube and incubated in a 37° C water bath for one hour. The reaction mixtures were centrifuged for one minute at 1500 rpm and read.

B. Coombs test:

(1) Cell suspensions were prepared as in A-1.

(2) 0.05 ml of serum and the same amount of antigen were placed in a 10 x 75 mm test tube and incubated in a 37° C water bath for one hour.

(3) The cells from (2) were washed three times with full tubes of saline. After the last washing, the saline was removed as completely as possible by flicking the tubes; 0.05 ml of anti-human globulin rabbit serum was added to the packed cells, the two being mixed thoroughly by vigorous manual agitation. The tests remained at room temperature for thirty minutes, were centrifuged at 1500 rpm for one minute, and read macroscopically.

C. Proteolytic enzyme (trypsin):

(1) Trypsin solution was prepared by dissolving 0.25 gm trypsin (Difco, 1:250) in 25 ml of 0.85% sodium chloride. After thorough shaking, the trypsin remaining undissolved was allowed to settle and the supernatant used for testing.

(2) A 2% red blood cell suspension was made in 0.85% sodium chloride solution, washed, and restored to volume in 0.15 molar phosphate-buffered saline at a pH of 7.1.

(3) 0.15 ml trypsin was used for each ml of 2% red blood cell suspension. The enzyme was allowed to react with the erythrocytes for ten minutes at 37° C. At the end of this period, the cells were washed once with saline and restored to volume in saline.

(4) 0.05 ml of serum and 0.05 ml trypsinized antigen were placed in a 10 x 75 mm test tube and incubated in a 37° C water bath for one hour before reading.

D. Polyvinylpyrollidone (PVP):

(1) A 2% red blood cell suspension was made in 0.85% sodium chloride solution, washed, and restored to volume in the same medium.

(2) 0.05 ml of serum and 0.15 ml of a 10% buffered solution of PVP (Holy Cross Hospital Research Foundation) were placed in a 10 x 75 mm test tube and mixed. 0.1 ml antigen was added and mixed. The serum-PVP-antigen mixture was centrifuged at 2500 rpm for two minutes and read immediately.

E. Serial dilutions:

(1) 0.1 ml of saline was placed in each of a series of 10 x 75 mm test tubes. 0.1 ml of serum was placed in the first tube, and

successive two-fold dilutions made in turn.

(2) 0.05 ml of red blood cell antigen, either trypsinized or in saline according to the particular test to be used, was added.

(3) The tubes were placed in a 37° C water bath for one hour before reading.

F. Absorption of serum:

(1) One ml of washed, packed erythrocytes was used to absorb two ml of anti-D serum.

(2) Absorption was allowed to proceed for one hour in a 37° C water bath, after which the tube was centrifuged at 4500 rpm for ten minutes and the supernatant serum removed as completely as possible with a Pasteur pipette drawn to a very fine tip.

G. Elution of Rh antibody:

(1) After the absorbed serum was removed in F-2, the cells were washed six times with full tubes of saline.

(2) 0.5 ml of saline was added to the washed cells, which were placed in a water bath at 56° C for fifteen minutes and agitated from time to time.

(3) The cells were centrifuged at 4500 rpm for ten minutes and the supernatant saline (usually deeply tinged with free hemoglobin) was removed and tested for Rh antibody.

PART II
Definition of a D^u Blood

In order that there be no ambiguity on this point, the following criteria have been established to identify a D^u blood specimen:

(1) gives a weak or negative reaction with one or more saline test tube anti-D serums;

(2) is agglutinable by one or more blocking anti-D serums;

(3) is capable of yielding anti-D antibody by elution after treatment with blocking anti-D serums.

The term "weak reaction" means one that appears negative or questionably positive macroscopically, but shows distinct agglutination when examined under the microscope.

PART III
Materials

Most of the materials used were carefully pretested laboratory reagents. Erythrocytes do not come into this classification, and the means for obtaining them as needed are described.

A. Rh typing serums:

(1) All typing serums were purchased from commercial sources.

(2) Preliminary studies.

Each anti-D typing serum was tested against a panel of D-negative cells containing as many antigens of other blood group systems as possible. Included were A, B, K, k, Le^a, Le^b, Lu^a, M, N, S, s, P, C, C^w, E, Fy^a, and Fy^b. The saline and Coombs tests were used for these determinations. None of the anti-D serums selected for use in any part of this report showed evidence of having antibody other than D.

(3) Titer.

The titer for each serum was determined by two-fold serial dilutions against a panel of ten D-positive cells, taking as the average titer the geometric mean. The saline and blocking serums chosen for investigation of incidence and classification of D^u bloods had an average titer of 1:128.

(4) Presence of antibody reacting with D^u erythrocytes.

(a) Blocking serums were tested against a small panel of twelve low-grade D^u bloods. The three picked to be employed in the incidence and classification of D^u bloods reacted with all twelve control specimens when the Coombs test was used.

(b) Saline serums were used without preliminary checking of D^u antibody content.

B. Specimens:

(1) For incidence and classification.

Samples used for this portion of our study were routine prenatal blood specimens submitted to the Blood Grouping Section of the Division of Laboratories, Utah State Department of Health. Careful checks were made to eliminate repeat or referred specimens, so the data reflect the findings for a random sampling of the population of Utah. None of the Rh factors are sex-linked, so the fact that the great majority of samples are from females does not detract from the validity of the figures presented.

(2) For preliminary absorption studies.

Most of the specimens were low-grade D^u bloods furnished through the kindness of Dr. Stanley Marcus and Mrs. Jessie Lamont of the Latter Day Saints' Hospital Blood Bank, Mr. Elmer Trentelman of the Holy Cross Hospital Research Foundation, and Mrs. Virginia Kreuzer of the American Red Cross Blood Service.

(3) For cross-reaction experiment.

A careful record was kept for each D^u blood specimen encountered from the period July 1, 1954, to March 1, 1955. At that time, a letter was sent to physicians of selected D^u patients explaining the uncertainty surrounding the D^u type and soliciting the cooperation of clinician and patient in obtaining a larger sample than that sent in for usual Rh work. A bottle containing sufficient ACD solution to prevent coagulation of eightly ml of

blood accompanied the letter. All of the specimens were received within a short period of time so that the cells could be stored in the refrigerator and tested at a later date with a number of absorbed serums.

PART IV
Incidence and Classification of D^u Bloods

Although considered separately, the same series of tests was employed for both phases of Part IV. In A, the incidence of all Rh types was of key importance, but in B the emphasis was on subdivisions of the D^u group.

A. Incidence.

Each specimen was tested with one anti-C, one anti-E, and three anti-D serums by the saline test tube technique. Weak reactors for D and negatives were examined with three anti-D blocking serums by the PVP, the proteolytic enzyme and the Coombs procedures.

B. Classification.

From the reaction pattern derived from IV-A, an attempt was made to classify the D^u blood specimens into distinct groups solely on the basis of the least sensitive techniques needed to demonstrate the D^u antigen. The provisional groups were:

D^u-I. D^u demonstrable by saline techniques;

D^u-II. D^u demonstrable by PVP test, not by saline;

D^u-III. D^u demonstrable by proteolytic enzyme test, not by saline or PVP;

D^u-IV. D^u demonstrable only by the Coombs test.

PART V
Preliminary Absorption Studies

A single anti-D blocking serum, HC_{10}, was employed in these tests. Two ml of serum were absorbed with one ml of washed, packed cells. After each absorption the serum was titrated against trypsinated D-positive erythrocytes and tested qualitatively (Coombs test) against the D^u cells being used to carry out the absorptions. An eluate prepared from the absorbing cells was also tested against trypsinated D-positive cells and the absorbing cells respectively. Checks of serum and eluate with the absorbing cells were carried out by the Coombs technique. Absorptions were carried out repeatedly until the criteria for complete removal of D^u antibody were met. The requirements were:

(1) failure to reduce the anti-D titer over at least three successive absorptions;

(2) failure to show anti-D antibody in the eluate over three successive absorptions; and

(3) failure to react with a suspension of the absorbing cells.

PART VI
Cross-Reactions Between D^u Bloods

Two ml of washed, packed cells from the specimens mentioned in Part III-B-2 were used to absorb one ml of anti-D blocking serum HC_{12}. Tests of absorbed serum and eluates were made as in Part V, except that no quantitative titrations were performed.

After all absorptions had been completed, each absorbed serum was tested with every D^u specimen used for absorption. The Coombs test was used throughout.

EXPERIMENTAL RESULTS

The presentation of experimental results follows the order outlined in Parts IV, V and VI of Materials and Methods.

It was necessary to determine the incidence of D^u before beginning any large-scale studies. If D^u blood specimens were found too infrequently, years might be spent gathering enough material to be representative. At the same time, a study of Rh blood types to detect deviations from previous figures for Caucasian populations, and to ascertain if D^u subtypes were associated more frequently with one Rh type than others, appeared reasonable. By using a combination of typing serums on the samples and testing each with the various methods, a means of classification might be worked out. Once it was established that the D^u subgroup appeared with a high enough frequency to make further studies feasible, preliminary investigations of the absorbing quality of D^u antigens were made as a guide to what might be expected when a large number of samples for cross-reaction experiments would have to be handled in a short period of time. The absorption and cross-reaction findings might also be correlated with classification.

PART I
Incidence of Rh Types

The data presented in Table 2 are felt to be a valid representation of the distribution of Rh types in Utah. This state has a very low percentage of Negro or other racial stocks which might increase or decrease the incidence of any particular type. The conformity of the findings to other studies on Caucasian populations makes it likely that the number of D^u specimens found is a true approximation of the frequency of this subtype in the white race.

The significant lack of D^u samples in the CDE group (Table 2, line 3) was difficult to rationalize until it was realized that over 90% of this group is homozygous for D. At the present time, there are no serological means for detecting the DD^u heterozygote, so all D^u blood specimens recognized must have the genotype D^uD^u or D^ud. The homozygote is rare enough to be a collector's item; therefore, it is likely that all of the D^u persoms found in this study were D^ud. Using the chromosome frequencies (or gene frequencies, if one employs the multiple allele hypothesis) presented by Race and Sanger (1950), the expected number of heterozygotes for D in each Rh type was determined and the percentage of D^u obtained for them. The percentage figures in the last column of Table 2 show that the number of persons carrying a chromosome containing a D^u gene is approximately the same in any Rh type where it is possible for it to occur.

The incidence of Cde and cdE is lower than that given in most previous studies. This is due to the fact that earlier work was done at a time when the only intermediate D types known were the ones giving weak reactions by saline methods. Little attention was paid to these reactions and they

Table 2

Rh TYPES OF 16,657 UNSELECTED BLOOD SPECIMENS

PHENOTYPE	NUMBER	PERCENTAGE	D^{u*}		% D^u	% D^u IN CALCULATED HETEROZYGOTES
			HIGH	LOW		
CDe	8628	51.80	111	66	2.05	3.08
cDE	2431	14.59	48	24	2.96	3.48
CDE	2383	14.31	6	2	0.34	2.90
cDe	362	2.17	6	6	3.31	3.42
Cde	85	0.51	0	0	-	-
cdE	101	0.61	0	0	-	-
cde	2667	16.01	0	0	-	-
CdE	0	0	0	0	-	-
TOTALS	16657	100.00	171	98	1.62	-

*High-grade: react with one or more of three saline anti-D typing serums. Most, if not all, would be considered D-positive in a majority of laboratories.

Low-grade: do not react with any saline typing serums. Requires PVP, proteolytic enzyme or Coombs test for detection.

were considered D-positive. Therefore, most, if not all, of the low-grade D^u bloods classified CDe in Table 2 would have been considered Cde, and the same holds true for cdE and CdE. Adding the low-grade CD^ue to the figures for Cde gives 85 / 66, or 151 apparent Cde, or 0.91%. Applying the same spurious correction to cdE yields 0.75%.

That D^u follows the distribution of D is shown by the fact that it occurs in connection with C, 43.7%, and with E, 19.7%, of the time.

The very rare phenotype CdE (estimated frequency 10^{-4}) was encountered twice. In both instances, however, the specimens gave D^u reactions and are included as the two low-grade members of the CDE type.

The saline-active, or high-grade, D^u type was found more frequently than low-grade, in a ratio of 1.75:1.

With the number of blood specimens received annually in this laboratory, the percentage of D^u samples, 1.62, was considered ample for continuing further studies.

PART II
Classification of D^u Specimens

The idea that a tentatively useful system of classification might be achieved on the basis of methodology grew from earlier observations (Argall, 1953) that the techniques available for detection of Rh antibody in serums varied widely in their sensitivity, the general order being proteolytic enzyme > Coombs test > PVP > albumin > slide > saline. This concept was tested by determining the reaction pattern of the 269 D^u blood specimens mentioned in Part I with three saline and three blocking anti-D typing serums, the latter being used with three different methods. It is obvious from Table 3 that the Coombs test is more reliable for detecting D^u bloods than is the proteolytic enzyme technique, thus reversing the order exhibited in Rh antibody testing. For screening large numbers of samples, the anti-human-globulin test would be the one of choice. PVP and saline follow these two tests in descending order of activity.

As a general rule, if a specimen reacts with any serum when a technique of lesser sensitivity is used, it will be agglutinated more strongly by the same serum, and usually by others as well, when a more sensitive procedure is applied. This is by no means invariable, however, for twenty-three exceptions are recorded in Table 3.

Three kinds of variation are to be noted. One has already been mentioned in the preceding paragraph and appears to be largely a result of the technique employed. A second kind of divergency is noted when the three serums are tested against the same D^u red blood cells by the same technique. Here the difference apparently lies in the qualitative antibody content of the serum, but there is no consistent pattern. The third

Table 3

REACTION PATTERN OF 269 D^u BLOOD SPECIMENS

METHOD	SALINE			PVP			TRYPSIN			COOMBS			NO.
TYPING SERUMS	HC_s	O_s	CB_s	HC_{12}	O	CB	HC_{12}	O	CB	HC_{12}	O	CB	
HIGH GRADE*	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	132
	≠	≠	-	≠	≠	≠	≠	≠	≠	≠	≠	≠	11
	≠	-	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	3
	-	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	≠	4
	-	≠	-	≠	≠	≠	≠	≠	≠	≠	≠	≠	4
	≠	-	-	≠	≠	≠	≠	≠	≠	≠	≠	≠	2
	≠	≠	-	-	-	-	≠	≠	-	≠	≠	≠	6
	-	≠	-	-	-	-	-	-	-	≠	≠	≠	6
	≠	≠	≠	-	-	-	≠	-	-	≠	≠	≠	3
LOW GRADE*	-	-	-	≠	≠	≠	≠	≠	≠	≠	≠	≠	8
	-	-	-	-	-	-	≠	≠	≠	≠	≠	≠	15
	-	-	-	-	-	-	≠	-	-	≠	≠	≠	35
	-	-	-	-	-	-	-	-	-	≠	≠	≠	24
	-	-	-	-	-	-	-	-	-	-	≠	-	4
	-	-	-	-	-	-	≠	-	-	≠	-	-	4
	-	-	-	-	-	-	≠	-	-	-	-	-	3
	-	-	-	≠	≠	-	-	-	-	≠	≠	≠	5

*See page 21.

appears to be related somehow to an effect produced by both method and serum in combination. It can be noted most clearly by comparing the HC_{12} and O typing serums. In trypsin, HC_{12} gives positive results with 230 samples, O reacts with only 185. Consideration of the Coombs test data shows that both serums agglutinate exactly the same number of D^u blood specimens, 262. One serum, then, may be much more effective than another with a given method, but no more so when another test is utilized.

PART III
Absorption Studies with D^u Samples

Before the absorption experiments were begun, the titer of typing serum HC_{12} was determined against each of the specimens used for removal of antibody. The serial dilutions were done in triplicate with the Coombs technique. The results of these tests are given in Table 4. The great variation exhibited by D^u cells is clearly evident, ranging from samples reactive with undiluted serum up to those giving the full titer expected of regular D-positive cells. It would appear that, for the most part, the limiting titer became lower in inverse ratio to the sensitivity of the method necessary to detect D^u, but the degree of overlapping makes the observation of dubious significance for practical purposes.

The data of Table 5 indicate that whereas D cells remove antibody swiftly and completely, D^u cells seem to remove small increments of antibody per absorption. Table 7 shows that many high-grade D^u samples can absorb all of the anti-D antibody, whereas others achieve only a marked reduction in titer. At the lowest end of the scale are samples of cells agglutinated weakly by the undiluted serum and capable of yielding small amounts of antibody on elution, but producing no demonstrable lowering of titer. The indication is that D^u erythrocytes can usually produce a diminution in the amount of anti-D antibody, but that the amount of antibody absorbed is a property of each individual sample.

When consideration is given to the action of the serum and that of the eluate after each successive absorption, a definite pattern may be observed, as illustrated in Tables 6 and 7. Almost invariably, the first tests to become negative are those made against the absorbing erythrocytes.

Table 4

TITER OF D^u CELLS WITH UNABSORBED ANTI-D SERUM, HC_{12} (COOMBS TEST)

GROUP	SPECIMEN	TITER 1: 1	2	4	8	16	32	64	128	256
D	CIA	----------	----------	----------	----------	----------	----------	----------	----------	----------
	FUL	----------	----------	----------	----------	----------	----------	----------	----------	----------
	RSF	----------	----------	----------	----------	----------	----------			
D^u-I	COO	----------	----------	----------	----------	----------	----------	----------	----------	----------
	MIL	----------	----------	----------	----------	----------	----------	----------		
	JOS	----------	----------	----------	----------	----------	----------	----------		
	AND	----------	----------	----------	----------	----------	----------	----------		
	THO	----------	----------	----------	----------	----------	----------	----------		
	BEN	----------	----------	----------	----------	----------				
D^u-II	PET	----------	----------	----------	----------	----------	----------	----------		
	REE	----------	----------	----------	----------	----------	----------	----------		
	BLO	----------	----------	----------	----------	----------				
	CLY	----------	----------	----------	----------	----------				
D^u-III	EVE	----------	----------	----------	----------	----------	----------	----------		
	KEV	----------	----------	----------	----------	----------	----------	----------		
	MAR	----------	----------	----------	----------	----------				
	RIC	----------	----------	----------	----------	----------				
	NOR	----------	----------	----------	----------	----------				
	RUS	----------	----------	----------	----------	----------				
	BAR	----------	----------	----------	----------					
	ANR	----------	----------	----------	----------					
	FOT	----------	----------	----------	----------					
D^u-IV	BUR	----------	----------	----------	----------					
	VAN	----------	----------	----------	----------					
	ALL	----------	----------	----------	----------					
	HAD	----------	----------	----------	----------					
	SAG	----------	----------	----------						
	ROB	----------	----------	----------						
	RC-13	----------	----------							
	RC-14	----------	----------							
ATYPICAL	COL	----------	----------	----------	----------					
	BOW	----------	----------	----------	----------	----------	----------	----------	----------	
	VAL	----------	----------	----------	----------	----------				

Table 5

ABSORPTION OF ANTI-D SERUM HC_{10} WITH D AND D^u ERYTHROCYTES

GROUP	ABSORBING ERYTHROCYTES	NUMBER OF ABSORPTIONS* 1	2	3	4	5	6	7	8	9	10	11	12
D	CIA	4[1]	1	0	0	0	0						
	5-2C	8	2	0	0	0	0						
	1-4B	4	2	0	0	0	0						
	2-8B	2	1	0	0	0	0						
	RSF	32	8	2	0	0	0	0					
D^u-I	JS	32	16	8	4	2	0	0	0	0			
D^u-II	JV	128	64	32	32	16	16	8	8	8	8	8	
	HC-1A	128	64	64	32	16	8	8	8	8	8	8	
D^u-III	VT	128	128	64	32	32	32	32	32	32			
	HG	128	128	64	32	32	32	32	32	32			
	HC-1	128	64	32	32	16	16	16					
	RC-1A	128	128	64	32	32	32	32					
	RC-2A	128	128	64	32	32	32	32	32				
	15	64	64	32	32	32	16	16	8	8	8	8	
	11	128	128	64	64	32	32	32	32				
	12	128	64	64	32	32	32	32	32	32			
	RC-2	128	128	64	64	64	64	64	64				
	RC-3	128	64	32	32	32	32	32	32				
	16	128	64	32	32	32	16	8	8	8	8	8	
	LD-1	64	64	64	32	32	32	32	32				
D^u-IV	RC-13	128	128	128	128								
	RC-14	128	128	128	128								
	DP	128	128	128	128								
	RC-1	128	128	64	64	64	64						
	LD-2	128	128	64	64	64	64	64					
	LD-3	128	128	128	128								

*Absorbed serum tested against pooled, trypsinated, D-positive erythrocytes.
[1]Figures indicate titers expressed as reciprocal of serum dilutions.

Table 6

ORIGINAL ABSORPTION AND ELUTION STUDIES WITH HC_{10}

GROUP	SPECIMEN USED FOR ABSORPTION	NO. OF ABSORPTIONS REQUIRED TO REMOVE ALL ANTIBODY[1]	NO. OF ABSORPTIONS REQUIRED TO REMOVE ANTIBODY FOR ABSORBING CELLS[2]	NO. OF ABSORPTIONS REQUIRED TO RENDER ELUATE NEGATIVE FOR D-POSITIVE CELLS[1]	NO. OF ABSORPTIONS REQUIRED TO RENDER ELUATE NEGATIVE FOR ABSORBING CELLS[2]	FINAL TITER OF ABSORBED SERUM
D	CIA	3	3	4	3	0
	5-2C	3	2	4	3	0
	1-4B	3	2	4	2	0
	2-8B	3	2	4	3	0
	RSF	4	3	5	3	0
D^u-I	JS	6	4	7	4	0
D^u-II	JV	*	4	9	4	8
	HC-1A	*	3	9	5	8
D^u-III	VT	*	4	7	5	32
	HG	*	3	7	4	32
	HC-1	*	3	5	2	16
	RC-1A	*	3	6	2	32
	RC-2A	*	3	6	3	32
	15	*	4	9	6	8
	11	*	3	6	3	32
	12	*	5	7	4	32
	RC-2	*	2	6	3	64
	RC-3	*	4	6	3	32
	16	*	4	9	3	8
	LD-1	*	4	6	2	32
D^u-IV	RC-13	*	2	2	2	128
	RC-14	*	1	2	2	128
	DP	*	1	2	1	128
	RC-1	*	2	4	2	64
	LD-2	*	3	5	2	64
	LD-3	*	2	3	2	128

*Never completely absorbed.

[1]Absorbed serum tested against pooled, proteolytic enzyme treated, D-positive cells

[2]Absorbed serum tested against absorbing cells by Coombs test.

Table 7

ABSORPTION OF SERUM HC_{12} WITH SPECIMENS USED IN CROSS-REACTION EXPERIMENT

GROUP	SPECIMEN USED FOR ABSORPTION	NO. OF ABSORPTIONS REQUIRED TO REMOVE ALL ANTIBODY[1]	NO. OF ABSORPTIONS REQUIRED TO REMOVE ANTIBODY FOR ABSORBING CELLS[2]	NO. OF ABSORPTIONS REQUIRED TO RENDER ELUATE NEGATIVE FOR D-POSITIVE CELLS[1]	NO. OF ABSORPTIONS REQUIRED TO RENDER ELUATE NEGATIVE FOR ABSORBING CELLS[2]	FINAL TITER OF ABSORBED SERUM
D	FUL	4	3	4	3	0
	CIA	3	3	4	3	0
D^u-I	MIL	7	3	6	3	0
	JOS	7	5	7	6	0
	COO	5	2	4	1	0
	AND	6	3	6	5	0
	BEN	*	4	6	5	0
	THO	*	6	7	6	2
D^u-II	PET	*	2	9	3	32
	REE	*	5	11	7	16
	BLO	*	6	8	6	4
	CLY	*	6	7	6	4
D^u-III	ANR	*	4	17	2	32
	MAR	*	3	9	4	32
	EVE	*	5	10	4	16
	RIC	*	6	8	4	2
	NOR	*	5	11	3	16
	KEV	*	4	11	3	16
	BAR	*	4	10	3	32
	FOT	*	4	13	3	32
	RUS	*	5	8	3	32
D^u-IV	ALL	*	3	13	3	32
	BUR	*	4	9	3	8
	SAG	*	3	4	2	64
	ROB	*	4	8	2	32
	VAN	*	7	9	6	8
	HAD	*	3	5	2	128
ATYPICAL	COL	*	6	8	4	4
	BOW	6	4	5	2	0
	VAL	*	2	5	3	8
cde	SHO					128

*Never completely absorbed.

[1]Absorbed serum tested against pooled, proteolytic enzyme treated, D-positive cells.

[2]Absorbed serum tested against absorbing cells by Coombs test.

Either may precede the other, although in any case they occur within one or two absorptions of each other. High-grade D^u blood cells which are capable of removing all anti-D antibody tend to yield negative eluates at about the same time that the serum no longer agglutinates D-positive red blood cells. Without exception, the eluates from all D and D^u blood specimens are positive against D erythrocytes for at least one absorption beyond the one in which no antibody for the absorbing cells can be demonstrated. Saline-inactive D^u erythrocytes almost invariably continue to yield specific anti-D antibody on elution long after both serum and eluate are negative for the absorbing cells.

The extreme case is that of the erythrocytes from patient ANR. After two absorptions, the eluate was negative for the ANR red cells; four absorptions were necessary to render the serum inactive; but anti-D antibody from the eluate continued to be demonstrable until the seventeenth absorption. This was such an unexpected occurrence that the whole experiment was repeated. On repetition, the eluate became negative for anti-D after one less absorption, but serum and eluate gave the identical results with the ANR cells that had been obtained initially. Based on the possibility that some unknown antibody was interfering, the specificity of the eluted antibody was checked after every five absorptions with D-positive and D-negative red cells. The reaction was specific for the D antigen in every instance.

The "atypical" cells listed in the last rows of Table 7 were placed in this category for definite reasons. The red blood cells of COL were saline-active, but required at least two hours for weak clumping to occur with two out of three serums. Trypsinized cells were agglutinated to about

the same extent, but the reaction took place within a half-hour and did not change appreciably on further standing. The PVP and Coombs tests were strongly positive with three undiluted blocking serums, although the titer was only 1:4 with blocking serums and 1:1 with the saline serums that would react at all. Despite the low titer, the absorbing power of this specimen was high. The red cells from BOW were agglutinated weakly with two saline serums, but so strongly by the third that it would have been classified as ordinary D. The PVP procedure was completely negative with three blocking serums, but by the other techniques, trypsin and Coombs, all three anti-serums displayed potent agglutinating power. The specimen from VAL was negative with all saline serums; positive with serum O alone in PVP; positive with HC_{12} alone in trypsin; positive with three blocking serums in the Coombs test. Despite these irregularities, COL and BOW behaved much like D^u-I specimens, while VAL was fairly typical of a D^u-II sample in the absorption and elution studies.

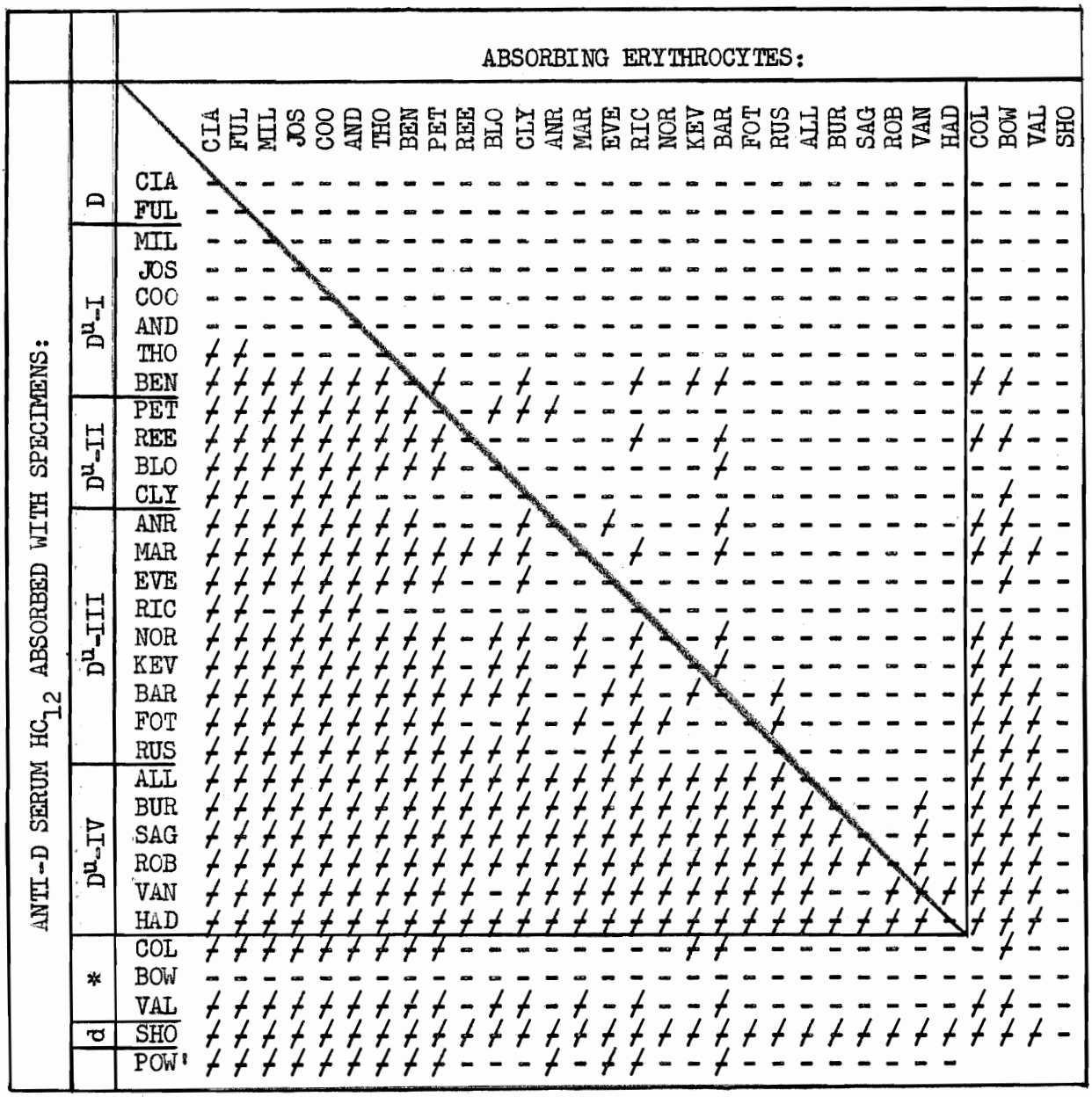
The cde controls were necessary to make certain that repeated heatings at 37^o C would not lower the serum titer non-specifically. It was also necessary to consider the possibility that small amounts of the saline used to wash the cells might be trapped in the packed column of erythrocytes to which the serum was added, thus diluting this reagent. The cde specimens were passed through eighteen absorptions using all techniques described for experiments with D^u cells. The failure to change the titer significantly indicated that the serum was not being unduly affected by extraneous factors.

PART IV
Cross-reaction Experiment

When the test cells are listed by the classificational grouping outlined previously and the serums absorbed with these erythrocytes are presented in the same arrangement, a general pattern may be observed. More positive reactions are found to the left of a diagonal drawn across Table 8; more negatives occur to the right. Usually, as the agglutinability diminishes, the cells are agglutinated less frequently by serums absorbed with erythrocytes lying above them on the scale. In turn, the cells appear to take less antibody out of the serum, hence the serum which they have absorbed most often agglutinates the erythrocytes from samples higher in the list. This is only a general phenomenon, however, because numerous exceptions are illustrated in the Table. In some instances, the results with individual serums and cells is quite orderly. For example, absorption of serum HC_{12} with ALL erythrocytes removes antibody against the other D^u-IV cells. The serums absorbed individually with these erythrocytes all give positive findings with the red cells from ALL. Similarly, SAG serum does not agglutinate ROB and HAD erythrocytes, while serums prepared from ROB and HAD do clump SAG cells. Most of the D^u-I specimens give concordant results, but BEN does not react like other members of the group. This patient appears to be transitional between D^u-I and D^u-II. Other exceptions occur, a good example being the cross-reaction between CLY and REE. In this instance, each sample completely removes antibody against the other, indicative of close or identical similarity in antigenic structure of the red blood cells. However, when the agglutination pattern of the same erythrocytes tested against serums

Table 8

CROSS-REACTIONS OF D^u SPECIMENS[1]



[1]Coombs test used throughout.
*Atypical samples. See text, page 45.
'Special serum from D^u patient who had developed anti-D antibody.

absorbed with the other specimens used in this study is considered, there are seven instances when a serum fails to agglutinate REE cells, but does react with CLY. Even more bizarre are the instances where two specimens exhibit mutual cross-absorption but the cells from one are agglutinated by a particular serum which does not agglutinate the other; again, other serums are encountered which give exactly the reverse results, e.g., BLO and ANR. These exceptions are numerous but do not invalidate the construction of a hypothesis concerning additive or step-wise accumulation of individual D^u antigens to make a complete D antigen.

DISCUSSION

Immunology has developed largely as an applied science based on empirical observations; its greatest concern has been the immediate problem of human or animal diseases, their prevention or cure. To the great majority of workers participating in this field, the basic question is "Does it work?", whereas the more fundamental questions of "Why" and "How" have been investigated by relatively few scientists. In its usual guise, immunology is a practical science almost divorced from the biological and physical sciences other than medicine.

However, no branch of science can remain pragmatic indefinitely, and this becomes especially true when pure research begins to replace the emphasis on practical application. Attempts to explain the facts and hypotheses derived from basic immunological experiments necessitate contact with other branches of science. The widening knowledge of human and animal blood group systems forces an immunologist to obtain a reasonable familiarity with genetics; conversely, the geneticist must know something about the fundamentals of immunology. There are many indications that serological data may be interpreted on a strictly chemical basis, quantitative and/or qualitative, and physical theories have been utilized for establishing hypotheses concerning antigen-antibody relationships. This overlapping of fields has resulted in the development of at least two poorly defined hybrids, the immunochemist and the immunogeneticist.

Any hypothesis elaborated to explain experimental results can not be firmly established unless the findings are based on reliable techniques and reagents of known composition. In addition, the possibility of human

error must be reduced to a minimum of reproducible results are to be obtained. Serological experiments must contend with all of these potential sources of error, and they must be considered in any interpretation of results.

The findings presented in this thesis indicate that about 1.62% of the members of a Caucasian population may be expected to have red cell antigens meeting the criteria established for classification as the intermediate Rh type, D^u. There are only two other studies of the incidence of D^u in a population. Renton (1950) found that 32% of specimens originally typed as Cde were actually CD^ue; 11% of cdE upon further checking were shown to be cD^uE. The frequency of the type cD^ue was estimated by Renton to be approximately 0.015%. A more complete study was made by Rosenfield, Vogel, Miller and Haber (1951) on a New York population. Their results showed that 44.7% of the time, Cde specimens were actually CD^ue; 20.6% of the cdE samples were more correctly classified as cD^uE. The incidence of cD^ue found by Rosenfield's group was over thirty times higher than that estimated by Renton, being 0.48%.

Besides making these comparisons with Renton's earlier data, the New York group studied the overall incidence of the D^u type, finding that 1.64% of all specimens could be placed in this category. High-grade D^u blood specimens outnumbered the low by a margin of 3:1. Rosenfield felt that technical differences did not account for the discordant results between the figures he compiled and those of Renton. Instead, he postulated that the discrepancy represents an effect due to the large number of persons of Eastern Mediterranean origin in New York City. The frequency of both cDe and cD^ue is higher in this group. This appeared to be a reasonable

explanation until an analysis was made of the incidence of the D^u type in Utah. The Rh types of the population of this state should correspond more closely to Renton's figures than to those of Rosenfield if the racial stocks comprising the population were the sole answer. Utah has an essentially Nordic population, the number of persons of other ethnical groups who might conceivably alter the ratios of the various Rh types being definitely low. In spite of this, the data presented could have been obtained from a New York Caucasian population, but could not conceivably be considered to resemble Renton's findings.

Essentially, the only marked discrepancy between the New York and Utah populations lies in the ratio of high to low-grade D^u specimens. This is probably due to a difference in definition. Rosenfield considers any sample agglutinated by a "one-stage" technique to be high-grade. By a one-stage test is meant the saline tube test with agglutinating serum, or a test (slide or tube) utilizing a blocking serum with albumin-suspended cells. By this criterion, all specimens in our series which react in saline, plus those reacting in PVP but not saline, would be high-grade. Our definition permits the classification of saline-active specimens alone in this group. If we add the PVP reactors to the 171 saline-active samples, the total high-grade reactors equals 184, low-grade 85; a ratio of 2.2:1. This approaches the 3:1 ratio obtained by Rosenfield.

There are a number of reasons to account for the fact that the actual percentage of the D^u subtype found is almost identical, despite the apparent difference in ethnical groups. One possibility lies in judgment. To be classified as a D^u specimen in Rosenfield's preliminary saline agglutinin test, a specimen had to give two-plus or less reactions. For our

purposes, a slightly weaker reaction than that encountered with the majority of erythrocytes was sufficient for calling a sample D^u. Differences of the order of magnitude of "one-plus" or "slightly weaker" are hardly quantitative in nature and permit room for enough error to account for any discrepancy in the number of D^u blood samples recorded. Theoretically, the figures obtained by Rosenfield should be higher than those reported here, so it is very likely that we were calling some samples D^u which were considered D-positive in Rosenfield's series. As justification for this practice on our part, it may be pointed out that the amount of agglutination obtained with these "almost-D" cells varied considerably when they were tested with additional serums. Nevertheless, the recognition of shaded changes in the amount of agglutination requires visual judgment and is open to some degree of subjective error.

The qualitative variation inherent in the serums themselves may also produce sufficient effect to alter the percentages of D^u specimens obtained from the same or similar populations by different investigators. Comparison of the saline serums used in this study shows that one identified 157, the second 166, and the third 152 out of 171 possible D^u specimens. Evidence furnished by Race, Sanger and Lawler (1948b) indicates that even greater discrepancies could be obtained if more serums were utilized. Thus, if our serums had a greater qualitative range, the net result would be the detection of more D^u specimens than would be possible with the single serum used in Rosenfield's study. On the other hand, the serum used by the latter investigator could have had a wider range than our three testing serums. The same reasoning applies to blocking serums, although the three used in this study showed minimal variation among themselves. It appears that our

serums did cover a wider range because more D^u specimens should have been found in New York if the assumption that the Eastern Mediterranean group was entirely responsible for the incidence of D^u were true. Rosenfield made no mention of what proportions of blood samples tested were actually from this group, so their influence may not have been as great as that presumed. No data were given for the incidence of the cDe type, which might have been a valuable clue.

The percentages found for the various Rh groups are in general agreement with those found by other investigators for supposedly Caucasian populations. This lends support to the belief that the incidence of D^u found is probably valid for such a group. Although actual figures may vary somewhat depending on the method used for detecting D^u and the interpretation placed on results with saline serums in particular, it appears reasonable to expect that the true incidence of the D^u subtype is between 1.5% and 2.0% for white populations.

While it has been demonstrated that D^u is antigenically active and can be responsible for transfusion reactions, either in providing the initial stimulus for the production of anti-D antibody or reacting with antibody already formed, the problem of eliminating D^u donors from the D-negative group is not as difficult as may appear. In the first place, the majority of high-grade D^u specimens will be disclosed by any of the methods utilizing blocking anti-D serums. If a Coombs test is run in addition, only a very few such samples will fail to give positive results with most commercial serums. While it is true that typing serums can vary greatly in the range of D^u which they are capable of agglutinating, any of the commercial products which we have employed appear to react with

most D^u samples, either high or low-grade. If a blood bank follows the practice of checking all apparently D-negative samples by the Coombs test, using a commercial blocking serum from a reliable manufacturer, only a rare D^u specimen will be overlooked. An alternate method is to type all D-negative donors with anti-C and anti-E serums, considering as technically Rh-positive all those reacting with either serum. This in itself will eliminate most D^u donors automatically, leaving only the very small number of low-grade cD^ue specimens undisclosed. The use of a large number of blocking serums from various sources, each checked as carefully as possible for anti-D^u content, would be an ideal procedure, but practically unsound in expenditure of time and money. A single good commercial blocking serum used with the sensitive Coombs test should be sufficient for resolving most problems apt to be encountered, although there must be a definite understanding that such a procedure cannot be expected to detect all of the weakly reacting subtypes of D. On the other side of the picture, recipients and pregnant women who fall into the D^u classification are potentially capable of forming anti-D antibody and for practical purposes must be considered D-negative. Strictly on a priori reasoning, it can be hypothesized that high-grade D^u persons would be less likely to produce anti-D antibody than those in the lower classifications. Placing such individuals in the D-positive group would not be too dangerous. It is apparent that the current problem is one of a reasonable compromise between the practical exigencies of laboratory life and the desire to make as few theoretically avoidable errors as possible.

The ability of one serum, HC_{12}, to cause visible agglutination of 42 D^u samples when the trypsin method was used, whereas the other serums

failed to produce this result, is an enigma. Perhaps part of the answer lies in the type of antibody present in this serum. Hill, Reid and Haberman (1949) have fractionated anti-D serums into three components, each having the same specificity but differing in mode of action and having different electrophoretic mobility. The saline type of agglutinin was found to be present in the gamma globulin, and blocking antibody (in the original sense of combining with, but not agglutinating D erythrocytes) in the albumin-euglobulin fraction. "Cryptagglutinoids" or antibodies requiring special methods for their detection, e.g., albumin suspension of cells, trypsin or Coombs test, were found in the beta globulin fraction. The concept of these investigators is that a reversible antigen-antibody reaction with competition between the various kinds of antibodies occur when they are present simultaneously in a serum. If this were the case, typing serum HC_{12} might be considered as being rich in cryptagglutinoids active against D^u components. Either antibody would yield agglutination of the red cells by the Coombs test, since the latter test is non-specific to the extent that any cells coated with human globulin will be agglutinated. Certainly further work is indicated on both of these problems--that of the kinds of antibody that may be present and that of the apparently aberrant results when serums and cells are the same and only the procedure is varied.

It has been intimated that various procedures with any given serum will show a definite order of activity in detection of D^u antigen, the number of negatives increasing in the order: Coombs test, proteolytic enzyme, PVP and saline, but that 23 out of 269 bloods failed to show this linear order. Of the individual serums tested, serums HC_{12} and O

exhibited 17 exceptions each; CB showed three. While proposing a method of classification that has an inherent error of 10-15% does not seem acceptable, the fact remains that it is the only system outlined to date which makes it possible to gain some insight into the serological reactivity of D^u cells. In no case in which D^u has been implicated in transfusion accident or erythroblastosis has there been any indication of the exact nature of the D^u types involved. It is still uncertain whether or not all grades of D^u are equally antigenic. It is suggested that the system of classification proposed might have a degree of usefulness, but that in any unusual case the D^u specimen concerned would have to be characterized as fully as possible by all available serums and methods.

When D^u cells are tested with serial dilutions of anti-D serums, the titers show a wide range. The highest titers are very close or identical to those of regular D cells; the lowest titers occur with undiluted serum only. That this is due strictly to the erythrocytes of each sample having varying quantities of the same antigen is unlikely. Even if only small amounts of antigen were present, it would seem that they should not exhibit such a wide range, but be more alike with regard to titer. On the basis of this analysis, the explanation of the results appears to reside, in part, in qualitative differences between D and D^u red cells, as well as between various D^u erythrocytes themselves. As previously noted, conjectures similar to these were used to postulate qualitative differences between the A_1 and A_2 antigens.

Absorption studies were also indicative of qualitative differences between D^u cells as well as between these cells and D cells. Erythrocytes belonging to the D group removed all agglutinins from serum HC_{12} rapidly

and completely. This was a consistent finding, capable of repetition with specimens other than the examples given in the tabulation of results. With the quantities of packed erythrocytes and serum used, no D samples have been encountered which required more than three absorptions before the serum ceased to react with all D and D^u cells. Many of the saline-active D^u specimens were capable of removing all antibody from the serum, although two were unable to accomplish this. One, THO, (Table 7) yielded an eluate with no antibody against D cells after seven absorptions, but the serum contained residual antibody capable of agglutinating D, but not D^u, cells. The second, BEN, followed a similar pattern, except that the residual antibody was anti-D $\neq$ D^u in nature. The rest of the specimens used in these experiments were incapable of removing all of the anti-D antibody; the remaining antibody agglutinated all D erythrocytes as well as varying numbers of D^u cells.

Further evidence for a qualitative difference between members of the D-D^u series was obtained when the serums absorbed with various D^u cells were tested against all members of the battery of specimens used in the cross-reaction study. The erythrocytes of three high-grade specimens, JOS, COO, and AND gave identical reaction patterns; two others, MIL and THO, were alike, although they differed from the first group. In no other cases were the cell patterns identical. The 28 D^u specimens included in this study appeared to represent 25 distinct subgroups of the subgroup D^u.

The final indication that qualitative differences exist between D^u specimens came from the tests made with the special serum, POW. The case from which this sample was obtained has been reported previously (Argall, Ball and Trentelman, 1953). The patient, whose serum contained anti-D antibody, belonged to a D^u subtype. The antibody reacted with a number

of D^u cells as well as with all D cells. This patient produced an apparently anti-D antibody capable of agglutinating D^u erythrocytes possessing structures which her own cells lacked. Unfortunately, it was not possible to obtain a specimen for inclusion in the absorption studies described.

In some cases, there appeared to be a quantitative difference between certain D^u cells. This was particularly noted with the specimen from patient ANR. (See page 45.) Her erythrocytes were apparently capable of removing only minute amounts of antibody during each absorption, yet enough antibody was absorbed so that after seventeen trials the anti-D titer was reduced by two tubes. The absorbed serum gave reactions with D^u cells in about the same proportion as serums absorbed by other members of the same classificational group. The results with specimen FOT resemble those of ANR. The erythrocytes of these two patients were agglutinated by few of the absorbed serums. This may be accounted for by presuming that the small amount of antibody left with which the cells could react and the few combining groups on these cells prevented visible agglutination, although antigen and antibody had reacted.

Many years ago, Landsteiner and van der Scheer (1936) showed that the injection of a simple chemical compound, e.g., serum protein diazonium para-aminobenzene sulfonic acid, could result in the formation of antibody against the compound. The clearest <u>in vitro</u> reactions were obtained with the antibody and its homologous antigen, but cross-reactions with chemical compounds of similar structure were found. Their interpretation was that the antibodies formed had their specificity determined by the whole molecule and not by any constituent part; although formed in response to a single antigen, the antibodies were not uniform but varied in specificity

to some degree. This variation in specificity was assumed to be responsible for cross-reactions with heterologous antigens.

Wiener (1943b) and Wiener and Wexler (1952) have adapted this concept to explain the serological and genetical facts concerning the known blood groups. The term "agglutinogen" means a structural part of an erythrocyte whose reality is inferred from its reactions with specific antiserums. The inheritance of an agglutinogen as a whole is taken to imply that it is a single structural unit. The unknown properties of an agglutinogen which determine its serological reactions are "serological factors". Even a simple antigen may have multiple serological factors; this is demonstrated by the cross-reactions mentioned in the preceding paragraph. The serological factors, according to Wiener, do not necessarily represent distinct chemical structures within the agglutinogen molecule, but may be only a pattern of electrical charges shared with other agglutinogens.

To exemplify this, the Rh agglutinogen C is assumed to be under the control of a single gene; agglutinogen CD is formed under the influence of a second allelomorphic gene. To account for the major Rh agglutinogens, a series of eight allelic genes is necessary. If the hypothesis is carried to its logical conclusion, each agglutinogen making up subtypes of C, D and E must be under control of still other modified alleles. The idea that a single locus can simultaneously control three factors is rather unique. As far as we know, the one gene-one enzyme theory has not been convincingly refuted as yet. (Lederberg (1951) interpreted certain results obtained with his Lac-3 mutant of Escherichia coli as evidence for true pleiotropy, but such examples are very rare.) Furthermore, the idea that a gene can influence a pattern of electrical charges which may not represent a physical entity

is rather difficult to grasp. Equally difficult to understand is the ability of three different genes to control the production of the same non-entity; e.g., the C (r'), CD (R^1) and CDE (R^2) genes all produce C agglutinogen, either alone or, in Wiener's concept, as a partial antigen within a complete agglutinogen.

The analysis described in the preceding paragraphs has been employed as an explanation of the serological findings with subgroup C^W. Race, Sanger and Lawlwer (1948c) suggested that the ability of either C or C^W erythrocytes to absorb anti-C + C^W serums free of both antibodies was due to linked antibody having two specific determinant groups on the same molecule, one against C and one against C^W. The C antigen was pictured as evoking anti-C antibody, and as an extra, completely non-specific response, anti-C^W on the same molecule. Wiener and Gordon (1949), in keeping with the agglutinogen hypothesis, state that anti-C + C^W serums really contain a single kind of antibody. C and C^W bloods possess structures of sufficient chemical similarity so that certain antiserums can cross-react between them. A pure anti-C serum not agglutinating C^W cells nor absorbed by them is considered evidence that C has a special serological property not possessed by C^W. This situation is analagous to that of the A_1 and A_2 subgroups. Both possess a component A in common. A_1 cells have a special property distinguishing them from A_2, the latter in turn having another property not shared with A_1, but common to A_2 and O agglutinogens. If this idea is assumed to be correct, an agglutinogen does not necessarily react as a whole molecule, as did the simple antigens of Landsteiner's experiments. This is clearly shown in considering the Rh type, CDe. The agglutinogen usually induces the formation of pure anti-D serum. If the molecule as a

whole were involved, the antibody would be expected to be anti-C and anti-D simultaneously, or more probably, a cross-reacting kind having an identity clearly distinguishable from anti-C and anti-D individually.

As applied to the D-D^u series, the mosaic hypothesis can explain many of the data. The simultaneous absorption of both D and D^u antibody by either kind of erythrocyte is indicative of similarity of structure. The weaker reactions given by D^u cells could be assumed to be due to the reaction of a heterologous antigen with an antiserum having a more distinct specificity for the homologous antigen, D. However, if all of the experimental observations were explainable on the basis of a single antibody, it would be expected that even the heterologous antigen could eventually absorb all of the antibody. Here again, the situation stands in a position analagous to that of the subgroups of A. Absorption of an ordinary anti-A serum with A_2 cells reduces the anti-A titer, but leaves behind an antibody residuum specific for A_1; specific in the sense that no visible agglutination of A_2 cells can be obtained. However, at least some A_2 cells can remove all of the antibody from an anti-A serum. The quantities of A_2 cells necessary to carry out this complete absorption were prodigious, as over twenty volumes of erythrocytes, washed and packed, were required to eliminate the antibody from one volume of serum. What ratios would be required for A_3, A_4, or A_5 cells are unknown, but it can be imagined that they would be much greater.

No attempt was made in the experiments reported here to determine whether all D^u cells were capable of absorbing an anti-D serum completely. The data of Table 5, however, furnish information that makes it appear unlikely that many of the specimens tested could accomplish complete

absorption. In this series of tests, approximately 0.15 ml of serum was removed after each absorption for determination of titer and other tests. Some samples required as many as nine to eleven absorptions before an eluate free of anti-D antibody was obtained. In these instances, less than 0.5 ml of serum remained. The one ml quantity of washed, packed cells was used throughout, even though the serum was steadily decreasing in volume. The total volume of erythrocytes was large as compared to the volume of serum and should have been sufficient to achieve complete absorption with at least some low-grade cells of groups D^u-II or D^u-III.

Since complete absorption could not be obtained with all D^u cells, it appears reasonable to assume that the blocking serums used in this study contained a population of antibodies having quite distinct specificities toward various D^u cells, but all capable of reacting with D erythrocytes. The other possibility, that a single antibody is present, seems less logical.

Conceivably, since the formation of antibodies reacting with heterologous cells occurs most noticeably after prolonged immunization, the broadening spectrum is due to a slow breaking down of the agglutinogen metabolically. If the component parts derived from this process were still antigenic, the antibody evoked might be considered as more homologous for D^u cells than for the original complete agglutinogen D.

The serums used in this study were produced as the result of stimulation with D erythrocytes, presumably containing a single agglutinogen. However, there has been no evidence established to warrant the tacit assumption that all agglutinogens thought to be D are exactly alike. If they are not, but possess many serological factors in common, the antibody

conglomeration produced against them would vary in individual specificities. If the agglutinogen happened to be lacking in a structure possessed by some D^u cells, no antibody would be formed against it and no reaction with the D^u cells could take place. Such a hypothesis could be employed to explain why serums differ in their ability to detect D^u cells even though their more obvious characteristics, titer, specificity for D cells, avidity, etc., are approximately equal.

The results found in the cross-reaction experiment tend to confirm, in a general way, the idea of a mosaic structure of D agglutinogens, with D^u cells being more or less complete replicas of the entire agglutinogen or portions of it. This is indicated by the larger number of positive reactions obtained when serums absorbed with low-grade D^u cells are tested against those of the same and higher grades; more negative tests are found as erythrocytes of increasingly higher grades are used. The exceptions to this generalization may be due to inherent technical error. For example, two specimens, CLY and RIC, apparently remove all antibody against each other from serum HC_{12}, yet their erythrocytes do not give identical reactions when tested with other absorbed serums. This seeming paradox may be due to the cells having identical structures in parts of their antigenic constituents and quite distinct structures in others. The residual antibody left after absorption with CLY cells may then combine with the erythrocytes of RIC but produce no visible reaction with the methods employed. This same residual antibody could conceivably agglutinate other D^u cells either because of a greater avidity or an increased quantity of the particular structure in them. It seems logical that a certain minimum amount of antibody must be combined with an antigen

before any obvious agglutination or precipitation will take place, so that if particular cells have less than a critical amount of antigenic surface of a given kind, they will fail to agglutinate. It should be mentioned that the majority of the reactions given in Table 8 were of sufficient strength to leave no doubt concerning reading, but some were very weak and required judgment for interpretation as positive or negative. Technical difficulties may have tended to obscure the pattern of reactivity demonstrated in Table 8 by creating more differences between individual samples than really existed. For this reason, the overall pattern is deemed to be more significant than the individual variations.

The concept of serological factors is of value in explaining the apparent multiplicity of antibodies formed against molecules of very simple antigens, but use of the term when referring to more complex antigens becomes an exercise in semantics. It has already been noted that parts of agglutinogens, e.g., C in the CD combination, may have identical serological factors in common with the C of Cde and CDE. It is not easy to consider them as anything but serologically demonstrable entities with the same chemical structure. It would follow, then, that the D agglutinogen may be looked upon as a mosaic structure composed of many antigenic groupings or serological factors while D^u agglutinogens are more or less complete replicas of parts of the overall mosaic of D. The antibodies formed in response to the D agglutinogen may be formed against the complete molecule or component parts of it. In essence, this condenses to the statement that linked antibodies may exist, although the term itself may not be a desirable one because of its genetic connotations. D^u cells would be capable of absorbing from a serum containing such linked antibody any

antibody with a grouping reactive with the particular complement of serological factors possessed by the D^u erythrocytes in question. The amount of apparently non-specific absorption would depend on the number of antibody molecules reactive for a paricular D^u specimen. Any remaining antibodies would be reactive with D and with other D^u erythrocytes possessing different groupings. The limtations of serological tests make it impossible to prove or disprove this concept with any degree of certainty at present.

Race, Sanger and Lawler (1948b) suggested very cautiously that certain results they obtained with D^u specimens indicated a stepwise series of additive gradations resulting from mutations. They did not consider the concept proved by their work. The material of this thesis confirms the idea in general, but different interpretations may be used to explain the source of these variations.

The subgroups of A and P exhibit the phenomenon of a continuously graded series of reactions. The C group of the Rh system is especially interesting because of c^v which is transitional between two entirely separate serological and genetical groups, C and c. It would seem possible to predict that when anti-d serum becomes freely available, some of the D^u specimens will be found to belong to a similar transitional group which might be designated d^v.

The stepwise gradations may be only an illusion created by limitations of serological techniques, and the true picture may be that of a smooth transition which could conceivably follow a normal curve. The majority of specimens reacting as typical D cells could be postulated to fall in the middle of the curve, the various gradations of D^u making up

one tail of the curve and "super-D" with very large numbers of serological factors constituting the other tail. From this it would follow that not all D specimens are identical, but contain enough antigenic groupings in common to give serologically indistinguishable reactions, at least with the vast majority of typing serums.

Theoretical considerations of this kind lead to the conclusion that neither the hypothesis of three linked genes nor the multiple allele concept is entirely correct. If multiple allelism exists at all at any of the Rh loci, it would appear to be associated with the subgroups, while the main antigens, C, D and E, represent closely linked genes. Just as plausible for explaining the findings associated with the D subgroups would be the interaction of genes which would not necessarily have to be linked. If each of the postulated interacting genes contributed to the production of a single antigenic grouping, but one master gene determined the way in which the individual antigens were to be combined in the complete agglutinogen, the linkage between C, D and E could be explained without recourse to multiple allelism. A second possibility is that the agglutinogens are built up in a single metabolic pathway, but that various modifying genes alter the normal procedure to produce incomplete, or subgroup, agglutinogens. Under this hypothesis, recombinations of various kinds could be expected; perhaps the case recorded by Race, Sanger and Lawler (1948b), in which one member of a family did not show the same kind of D^u as did the others, is the first example. More definite results may come from further investigations in this field.

After a lapse of seven years, the words of the British group of workers, "there is much to be learnt", are still valid. In any event, it

seems that no hypothesis with regard to human blood groups should be defended too vehemently until further data are forthcoming.

SUMMARY

1. The incidence of detectable Rh intermediate types, collectively classified as D^u, is estimated to be between 1.5% and 2% for a Caucasian population. This is in agreement with the only other reported large-scale investigation.

2. Absorption of an anti-D serum with D^u erythrocytes usually results in lower titers for both D and D^u cells, indicating a degree of serological relationship between these agglutinogens. Many D^u cells can remove all anti-D $\neq$ D^u antibody; others leave behind a residual antibody or antibodies specific for D and often for other D^u cells as well.

3. Evidence from absorption, elution and titration studies support the hypothesis that most D^u cells differ qualitatively from each other and are also qualitatively different from D erythrocytes. Quantitative variations are also observed. In most cases the qualitative aspects are the most prominent.

4. When D^u erythrocytes are used as absorbing reagents, failure of the absorbed serum to react with the absorbing cells is a poor criterion that removal of antibody is as complete as possible with the particular cells. These cells are usually capable of yielding eluates containing anti-D antibody long after the serum gives no visible agglutination with the absorbing cells.

5. Suggestions are made for the detection of D^u specimens in practical work. The data furnish a reasonable basis for predicting how many D^u samples will be overlooked if single anti-D blocking serums are used with various techniques.

6. The classification of D^u specimens into grades based on the

least sensitive method required to detect them is made. Probably due to serological limitations, the method has an error of approximately 12%. This is too large to be practical. It is suggested that in the absence of a reliable procedure for classification, unusual cases involving D^u antigens should include a detailed description of the serological activity of the D^u subtype.

7. When serums absorbed with D^u erythrocytes are tested against other D^u cells, a general pattern may be observed. The serologically less reactive samples usually remove smaller increments of antibody than do the more reactive ones, and serums absorbed by them tend to cross-react with larger numbers of D^u specimens. Conversely, cells of greater serological activity are agglutinated more frequently by serums absorbed with lower grade cells; serums absorbed with cells of higher activity show decreased tendency to agglutinate cells of lower order. Erythrocytes containing ordinary D agglutinogen appear to be capable of removing all antibody from anti-D serums.

8. It is suggested that the genetical activity at the Rh loci may be more complex than indicated by the multiple allele or linked gene theories. Interaction of genes (quantitative inheritance) or the influence of modifying genes may be more plausible for explaining many of the serological data. If multiple allelism exists at all, it is probably concerned with subgroups at a particular locus.

REFERENCES

Andresen, P. H. 1947 Reliability of the exclusion of paternity after the MN and ABO systems as elucidated by 20,000 mother-child examinations and its significance to the medico-legal conclusion. Acta Path. et Microbiol. Scand. 24:545.

Argall, C. I. 1951 Unpublished observations.

Argall, C. I. 1953 Master's Thesis, Stanford University.

Argall, C. I., Ball, J. M., and Trentelman, E. 1953 Presence of anti-D antibody in the serum of a D^u patient. J. Lab. and Clin. Med. 41:683.

Armytage, M., Cepellini, R., Ikin, E. W., and Mourant, A. E. 1950 A new allele of the Rh gene E. Bull. Ist. Siero. Milanese. In press. (original not seen, cited from Race and Sanger, 1950).

Brown, H. R. Jr., and Levine, P. 1943 Rh factor and its importance in transfusion for anemias of erythroblastosis and other causes. J. Pediat. 13:290.

Burnham, L. 1941 The common etiology of erythroblastosis and transfusion accidents in pregnancy. Am. J. Obstet. and Gynec. 42:389.

Callender, S. T., and Race, R. R. 1946 Serological and genetical study of multiple antibodies formed in response to blood transfusion by patient with lupus erythematosis diffusus. Ann. Eugenics 13:102.

Choremis, C., Ikin, E. W., Lehmann, H., Mourant, A. E., and Zannos, L. 1953 Sickle cell trait and blood groups in Greece. Lancet 2:909.

Coca, A. F., and Klein, H. 1923 A hitherto undescribed pair of isoagglutination elements in human beings. J. Immunol. 8:477.

Collins, J. O., Sanger, R., Allen, F. H., and Race, R. R. 1950 Nine blood group antibodies in a single serum after multiple transfusions. Brit. Med. J. 2:1297.

Coombs, R. R. A., Mourant, A. E., and Race, R. R. 1945 A new test for the detection of weak and "incomplete" Rh agglutinins. Brit. J. Exper. Path. 30:255.

Crome, W. 1935 Über Blutgruppenfragen: Mutter M, Kind N. Deut. Ztschr. f. d. ges. gerichtl. Med. 24:167.

Culbertson, C. G., and Ratcliffe, A. W. 1936 Reaction following intragroup blood transfusion; irregular agglutinin demonstrated by sensitive centrifuge test method. Am. J. Med. Sci. 192:471.

Darrow, R. R. 1938 Icterus gravis (Erythroblastosis neonatorum). Arch. Path. 25:378.

Davidsohn, I. 1937 A method for recognition of blood subgroups A_1 and A_2. J. A. M. A. 112:713.

Davidsohn, I., and Toharsky, B. 1942 The Rh blood factor. An antigenic analysis. Am. J. Clin. Path. 12:8.

von Decastello, A., and Sturli, A. 1902 Über die isoagglutinine im serum gesunder und kranker menschen. München med. Wchnschr. 49:1090.

DeGowin, E. L., and Baldridge, C. W. 1934 Fatal anuria following blood transfusion. Inadequacy of present tests for compatibility. Am. J. Med. Sci. 188:555.

Diamond, L. K., and Abelson, N. M. 1945 The demonstration of anti-Rh agglutinins. An accurate and rapid slide test. J. Lab. and Clin. Med. 30:204.

Diamond, L. K., and Allen, F. H. 1949 Rh and other blood groups. New England Med. J. 241:867.

Diamond, L. K., and Denton, R. L. 1945 Rh agglutination in various media with particular reference to the value of albumin. J. Lab. and Clin. Med. 30:821.

Dienst, A. 1905 Das Eklampsiegift: vorlaufige Mitteilung. Zentralbl. f. Gynäk. 29:353.

von Dungern, E., and Hirszfeld, L. 1911 Über gruppenspezifische Strukturen des Blutes. Ztschr. f. Immunitäts, 8:526.

Ehrlich, P. 1910 Studies in Immunity. John Wiley and Sons, New York, New York. 712 pp. Translated by C. Bolduan.

Fischer, W., and Hahn, F. 1935 Über auffallende Schwache der gruppenspezifischen Reaktionsfähigkeit bei einem Erwachsenen. Ztschrf. f. Immunitäts. 84:177.

Fisher, R. A. 1944 Cited By Race, R. R. An incomplete antibody in human serum. Nature 153:771.

Fisk, R. T., and McGee, C. A. 1947 The use of gelatin in Rh testing and antibody determinations. A rapid test tube method. Am. J. Clin. Path. 17:737.

Formaggio, T. G. 1950 Tecnica per l'attivazione degli anticorpi Rh nincompeti mediante l'uso di derivitavo polivenilico. Sangue (Milano) 23:243.

Friedenreich, V. 1936a Über einen eigentumlichen A-Receptor-Typus und seine Erblichkeitsverhaltnesse. Ztschr. f. Immunitäts. 89:409.

Friedenreich, V. 1936b Ein erblicher defekter N-Rezeptor der wahrscheinlich eine bisher unbekannte Blutgruppeneigenschaft innerhalb des MN-Systems darstellt. Deut. Ztschr. f. d. ges. gerichtl. Med. 25:358.

Friedenreich, V., and Lauridsen, A. 1938 On variety of human-type antigen M and its relation to other M antigens. Acta Path. et Microbiol. Scand. 38:155.

Friedenreich, V., and With, S. 1933 Über B-Antigen und B-Antikörper bei Menschen und Tieren. Ztschr. f. Immunitäts. 78:152.

Gammelgaard, A., and Marcussen, P. V. 1940 Nachweis eines vierten allelomorphen A-gens (A_4). Ztschr. f. Immunitäts. 98:411.

Goudemand, M., and Samaille, J. 1950 L'utilisation du polyvinyl pyrrolidone et le test à la trypsine dans la recherche des anticorps anti-Rhesus. Étude comparative avec d'autres techniques. Ann. Inst. Pasteur Lille 3:127.

Grove-Rasmussen, M., Soutter, L., and Levine, P. 1952 A new blood subgroup (A_0) identifiable with group O serums. Am. J. Clin. Path. 22:1157.

Grubb, R. 1949 Dextran as a medium for demonstration of incomplete anti-Rh agglutinins (Preliminary report). J. Clin. Path. 2:223.

Guthrie, C. G., and Huck, J. G. 1923 On the existence of more than four isoagglutinin groups in human blood. Bull. Johns Hopkins Hosp. 34:37.

Henningsen, K. 1949 Investigations on the blood factor P. Acta Path. et Microbiol. Scand. 26:639.

Hill, J. M., and Haberman, S. 1948 Two examples of sera containing the anti-d agglutinin predicted by Fisher and Race. Nature 161:688.

Hill, J. M., Haberman, S., Guy, R., and Davenport, J. W. Jr. 1950 Additional evidence for the anti-d: Statistical studies. Blood 5:1049.

Hill, J. M., Reid, A. F., and Haberman, S. 1949 The Rh antibody problem. Differentiation of agglutinins, agglutinoids, and cryptagglutinoids. Texas St. J. Med. 45:477.

Hirszfeld, L., and Amzel, R. 1940 Sur les formes de transition du groupe A. Rev. d'Immunol. 6:31.

Jakobowicz, R., Bryce, L. M., and Simmons, R. T. 1949 The occurrence of unusual positive Coombs reactions and M variants in the blood of a mother and her first child. Med. J. Australia 36:945.

Javert, C. T. 1942 Further studies on erythroblastosis neonatorum of obstetric significance. Am. J. Obstet. and Gynec. 43:921.

Jones, R. A. 1950 Dextran as a diluent for univalent antibodies. Nature 165:118.

Jones, A. R., Steinberg, A. G., Allen, F. H., and Diamond, L. K. 1953 Report of a second example of the Rh agglutinogen c^{v}, with some comments on its relation to the agglutinogen f. Science 118:721.

Landsteiner, K. 1900 Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe. Zentralbl. f. Bakteriol., Parasitenk. u. Infektionskr. 27:351.

Landsteiner, K. 1901 Über Agglutinationserscheinungen normalen menschlichen Blutes. Wien. klin. Wchnschr. 14:1132.

Landsteiner, K., and Levine, P. 1926 On the cold agglutinins in human serum. J. Immunol. 12:441.

Landsteiner, K., and Levine, P. 1927a A new agglutinable factor differentiating individual human bloods. Proc. Soc. Exper. Biol. and Med. 24:600.

Landsteiner, K., and Levine, P. 1927b Further observations on individual differences of human blood. Proc. Soc. Exper. Biol. and Med. 24:941.

Landsteiner, K., and Levine, P. 1928 On individual differences in human blood. J. Exper. Med. 47:757.

Landsteiner, K., and van der Scheer, J. 1936 On cross reactions of immune sera to azoproteins. J. Exper. Med. 63:339.

Landsteiner, K., and Wiener, A. S. 1937 On presence of M agglutinogens in blood of monkeys. J. Immunol. 33:19.

Landsteiner, K., and Wiener, A. S. 1940 An agglutinable factor in human blood recognized by immune sera for Rhesus blood. Proc. Soc. Exper. Biol. and Med. 43:223.

Landsteiner, K., and Wiener, A. S. 1941 Studies on an agglutinogen (Rh) in human blood reacting with anti-Rhesus sera and human isoantibodies. J. Exper. Med. 74:309.

Landsteiner, K., and Witt, D. H. 1926 Observations on the human blood groups. Isoagglutinins in sera of group IV. The factor A_1. J. Immunol. 11:221.

Lattes, L. and Cavazzuti, A. 1924 Sur l'existence d'un troisiéme élément d'isoagglutination. J. Immunol. 9:407.

Lawler, S. D., and van Loghem, J. J. 1947 The Rhesus antigen C^{w} causing hemolytic disease of the newborn. Lancet 2:545.

Lederberg, J. 1951 Chapter XIII, Genetics in the 20th Century. 634 pp., MacMillan Co., New York, New York.

Levine, P. 1941 Yearbook of Pathology and Immunology. pp. 508-513.

Levine, P. 1942a Erythroblastosis fetalis and other manifestations of isoimmunization. West. J. of Surg., Obstet., and Gynec. 50:468.

Levine, P. 1942b The Rh factor in erythroblastosis and transfusion accidents. Proc. N. Y. State Assoc. Pub. Health Lab. 22:42.

Levine, P. 1943a Pathogenesis of erythroblastosis fetalis, a review. J. Pediat. 23:656.

Levine, P. 1943b Pathogenesis of erythroblastosis fetalis and related conditions, Cincinnatti Med. J. 23:596.

Levine, P. 1946 The present status of the Rh factor. Am J. Clin. Path. 16:597.

Levine, P., Burnham, L., Katzin, E., and Vogel, P. 1941 The role of isoimmunization in the pathogenesis of erythroblastosis fetalis. Am. J. Obstet. and Gynec. 42:925.

Levine, P., and Katzin, E. M. 1940 Isoimmunization in pregnancy and varieties of isoagglutinins observed. Proc. Soc. Exper. Biol. and Med. 45:343.

Levine, P., Katzin, E. M., and Burnham, L. 1941 Isoimmunization in pregnancy; its possible bearing on the etiology of erythroblastosis fetalis; statistical evidence. J. A. M. A. 116:285.

Levine, P., Kuhmichael, A. B., Wigod, M., and Koch, E. 1951 A new blood factor, s, allelic to S. Proc. Soc. Exper. Biol. and Med. 78:218.

Levine, P., and Stetson, R. E. 1939 An unusual case of intragroup agglutination. J. A. M. A. 113:126.

Levine, P., Vogel, P., Katzin, E., and Burnham, L. 1941 Pathogenesis of erythroblastosis fetalis; statistical evidence. Science 94:371.

van Loghem, J. J. 1947 Production of Rh agglutinins anti-C and anti-E by artificial immunization of volunteer donors. Brit. Med. J. 2:958.

van Loghem, J. J., Bartels, H. L. J. M., and van der Hart, M. 1949 La production d'un anticorps anti-C^w par immunisation artificielle d'un donneur bénévole. Rev. d'Hem. 4:172.

McNeil, C., and Trentelman, E. F. 1952a I. Detection of Rh antibodies by polyvinylpyrrolidone (PVP). Am. J. Clin. Path. 22:77.

McNeil, C., and Trentelman, E. F. 1952b Detection of antibodies by polyvinylpyrrolidone. II. Comparison of sensitivity of anti-human-globulin and PVP. Proc. Soc. Exper. Biol. and Med. 78:674.

Mollison, P. L. 1949 Unpublished observations, cited by Stratton and Renton, 1949.

Morton, J. A., and Pickles, M. M. 1947 Use of trypsin in the detection of incomplete Rh antibodies. Nature 159:779.

Mourant, A. E. 1945 New Rhesus antibody. Nature 155:542.

Mourant, A. E., Ikin, E. W., Hässig, A., Hässig, R., and Holländer, L. 1952 Über das Vorkommen des Rhesusgens E^u in einer Ostschweizer Familie. Schweiz. med. Wchnschr. 82:1100.

Neter, E. 1936 Observations on abnormal isoantibodies following transfusions. J. Immunol. 30:255.

Parsons, L. G., Hawksley, J. C., and Gittins, R. 1933 Studies in anemias of infancy and early childhood; hemolytic (erythronoclastic) anemias of neonatal period, with special reference to erythroblastosis of newborn. Arch. Dis. Childhood 8:159.

Pietrusky, F. 1940 Zur vererbung der Blutgruppeneigenshaft N_2. Ztschr. f. Immunitäts. 98:387.

Potter, E. L., Davidsohn, I., and Crunden, A. B. 1943 Importance of Rh blood factor in erythroblastosis. Am. J. Obstet. and Gynec. 45:254.

Prokop, O. 1951 Über den Gelatin-Rhesustest. Ztschr. f. d. ges. gericht. Med. 40:192.

Race, R. R. 1944 An "incomplete" antibody in human serum. Nature 153:771

Race, R. R., and Sanger, R. 1950 Blood Groups in Man. 1st edition, 290 pp., Charles C. Thomas, Springfield, Ill.

Race, R. R., Sanger, R., and Lawler, S. 1948a Rh genes allelomorphic to C. Nature 161:316.

Race, R. R., Sanger, R., and Lawler, S. 1948b The Rh antigen D^u. Ann. Eugenics 14:171.

Race, R. R., Sanger, R., and Lawler, S. 1948c Allelomorphs of the Rh gene C. Heredity 2:237.

Race, R. R., Sanger, R., and Lawler, S. 1948d Rh genes allelomorphic to D. Nature 162;292.

Race, R. R., and Taylor, G. L. 1943 A serum that discloses the genotype of some Rh positive people. Nature 152:300.

Race, R. R., Taylor, G. L., Boorman, K. E., and Dodd, B. S. 1943 Recognition of Rh genotypes in man. Nature 152:563.

Renton, P. H. 1950 The Rhesus factor D^u. Thesis, University of Manchester, cited by Race and Sanger, 1950.

Rosenfield, R. E., Vogel, P., Gibbel, N., Sanger, R., and Race, R. R. 1953 A "new" Rh antibody, anti-f. Brit. Med. J. 1:975.

Rosenfield, R. E., Vogel, P., Miller, E. B., and Haber, G. 1951 Weakly reacting Rh-positive (D^u) bloods. Blood 6:1123.

Sanger, R., and Race, R. R. 1947 Subdivisions of the MN blood groups in man. Nature 160:505.

Schütze, H. 1921 Hemagglutination and its medico-legal bearing, with observations upon the theory of isoagglutinins. Brit. J. Exper. Path. 2:26.

Stratton, F. 1946 A new Rh allelomorph. Nature 158:25.

Stratton, F., and Renton, P. H. 1948 Rh genes allelomorphic to D. Nature 162:292.

Stratton, F., and Renton, P. H. 1949 The Rhesus factor D^u, a note on its practical importance. Brit. Med. J. 2:682.

Unger, L. J. 1921 Precautions necessary in the selection of a donor for blood transfusion. J. A. M. A. 76:9.

Walsh, R. J., and Montgomery, C. 1947 A new human isoagglutinin subdividing MN blood groups. Nature 160:504.

Wiener, A. S. 1938 The agglutinogens M and N in anthropoid apes. J. Immunol. 34:11.

Wiener, A. S. 1941a Subdivisions of group A and group AB; isoimmunization of A_2 individuals against A_1 blood, with special reference to the role of subgroups in transfusion reactions. J. Immunol. 41:181.

Wiener, A. S. 1941b Hemolytic reactions following transfusions of blood of the homologous group. II. Further observations on the role of the property Rh, particularly in cases without demonstrable antibodies. Arch. Path. 32:227.

Wiener, A. S. 1942 Hemolytic transfusion reactions. III. Prevention, with special reference to the Rh and cross-match tests. Am. J. Clin. Path. 12:302.

Wiener, A. S. 1943a Distribution and heredity of variants of the Rh types. Science 98:182.

Wiener, A. S. 1943b Genetic theory of the Rh blood types. Proc. Soc. Exper. Biol. and Med. 54:316.

Wiener, A. S. 1943c Blood Groups and Transfusions. 3rd edition, 438 pp., Charles C. Thomas, Springfield, Ill.

Wiener, A. S. 1944a Nomenclature of the Rh blood types. Science 99:532.

Wiener, A. S. 1944b The Rh series of allelic genes. Science 100:595.

Wiener, A. S. 1944c A new test (blocking test) for Rh sensitization. Proc. Soc. Exper. Biol. and Med. 56:173.

Wiener, A. S. 1945a Theory and nomenclature of the Hr blood factors. Science 102:479.

Wiener, A. S. 1945b Conglutination test for Rh sensitization. J. Lab. and Clin. Med. 30:662.

Wiener, A. S. 1946a Note on the pathogenesis of erythroblastosis fetalis. Am. J. Clin. Path. 16:319.

Wiener, A. S. 1946b Pathogenesis of erythroblastosis fetalis. Am. J. Clin. Path. 16:761.

Wiener, A. S. 1946c Theory and nomenclature of Rh types, subtypes and genotypes. Brit. Med. J. 1:982.

Wiener, A. S. 1946d Pathogenesis of congenital hemolytic disease (erythroblastosis fetalis). I. Theoretic considerations. Am. J. Dis. Childhood 71:14.

Wiener, A. S. 1950 Present status of Rh-Hr blood types. Brit. Med. J. 1:813.

Wiener, A. S., and Brancato, G. 1952 Problems in the management of erythroblastosis fetalis. J. Lab. and Clin. Med. 40:27.

Wiener, A. S., and Gordon, E. B. 1949 Studies on the blood factor rh^{W}. Am. J. Clin. Path. 19:621.

Wiener, A. S., and Landsteiner, K. 1943 Heredity of variants of the Rh type. Proc. Soc. Exper. Biol. and Med. 53:167.

Wiener, A. S., and Peters, H. R. 1940 Hemolytic reactions following transfusions of homologous group, with 3 cases in which some agglutinogen was responsible. Ann. Int. Med. 13:2306.

Wiener, A. S., and Sonn, E. B. 1943 Additional variants of the Rh type demonstrable with a special human Rh serum. J. Immunol. 47:461.

Wiener, A. S., Sonn, E. B., and Belkin, R. B. 1943 Heredity and distribution of the Rh blood types. Proc. Soc. Exper. Biol. and Med. 54:238.

Wiener, A. S., Sonn, E. B., and Belkin, R. B. 1944 Heredity of the Rh blood types. J. Exper. Med. 79:235.

Wiener, A. S., Sonn, E. B., and Polivka, H. 1946 Heredity of the Rh blood types. V. Improved nomenclature. Additional family studies with special reference to Hr. Proc. Soc. Exper. Biol. and Med. 61:382.

Wiener, A. S., Unger, L. J., and Sonn, E. B. 1945 New data on distribution of Rh blood types. Proc. Soc. Exper. Biol. and Med. 58:89.

Wiener, A. S., and Wexler, I. B. 1952 The mosaic structure of red blood cell agglutinogens. Bact. Revs. 16:69.

Witebsky, E. 1948 Interrelationship between the Rh system and the ABO system. Blood 3, suppl.2:66.

Zacho, A. 1936 "Unverträglichkeit" zwischen Blutproben vom gleichen Bluttypus beruhend auf dem Vorhandsein eines irregulären Agglutinins gegenuber einem bisher unbekannten Rezeptor. Ztschr. f. Rassenphysiol. 8:1.