THE QUESTION OF THE Rh HAPTEN

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The claim of Carter (1949) to have isolated from D-positive red cells a hapten-like substance reacting specifically with anti-D sera aroused universal interest. From Rhesus-positive erythrocytes she extracted with ether a piloid substance soluble in alcohol. An emulsion in saline of this solution gave a positive complement fixation test with anti-D sera and also caused inhibition of such sera. She further reported the clinical use of this substance which, when injected, caused a fall in the Rh agglutinins in sensitized pregnant women. There has, however, been no confirmation of this work from any other source, though some have failed to substantiate the original claims (Coombs, 1950; Stratton and Renton, 1950). The practical value of such a substance seemed sufficiently obvious to justify an investigation of the problem if only to indicate some of the difficulties involved.

Landsteiner and von Eisler (1904) first showed that petroleum ether extracts of animal red cells had an inhibitory effect on haemolytic antisera prepared from the same species of cell. Balls and Korns (1918) failed to demonstrate any specific properties in the lipoids extracted from sheep cells.

Sachs and Klopstock (1925) first showed that such substances as lecithin were antigenic when injected together with a foreign protein. Van der Scheer, Levene, and Landsteiner (1927) were able to confirm this, using commercial ovo-lecithin. Landsteiner concluded that the positive results were due to the presence of impurities in the ovo-lecithin, and there have subsequently been many conflicting reports on the antigenicity of lecithin.

Landsteiner and van der Scheer (1925) obtained alcoholic extracts from red cells of various species, and their extracts corresponded fairly closely to the first filtrate in Carter's method. They found that horse blood extracts would not inhibit immune sera prepared against the whole blood, from which one may fairly conclude that such extracts do not necessarily contain all the antigenic components of the cells.

Belkin and Weiner (1944) showed that the red cell stroma possessed Rh specificity, and subsequently Calvin, Evans, Behrendt, and Calvin (1946) reported the existence of an ether-soluble fraction of the stroma which also had Rh specificity. This fraction they called elinin, and the work of these latter authors is the basis of the experiments reported by Carter.

Spielmann (1949) showed that alcoholic extracts purified by acetone precipitation would inhibit all antibodies of the Rh system, the effect being similar for anti-D, anti-C, and anti-c.

Progress in the isolation of the other blood group substances has been greatly facilitated by their occurrence in water-soluble form, and a considerable amount is known about them from a chemical point of view, but of the Rh antigens, so far unconfirmed in body fluids, we know nothing.

Materials and Methods

The Cell Extracts.—The method described by Carter (1949) was carefully followed. The "positive" extract was obtained from four D-positive donors, the genotypes being cDE/cde (3) and CDe/cDE (1). The "negative" extract was similarly obtained from four D-negative donors (cde/cde). All donors were group A. After ether extraction the solvent was removed by distillation under reduced pressure, the temperature never rising above 30° C., and the residue finally dried over phosphorus pentoxide with a high vacuum pump. A 1% solution in ethyl alcohol was made, and this appeared to be almost saturated at room temperature.

Inhibition Tests.—These were carried out by the following technique :

Doubling dilutions of serum were set up in two or more rows with saline controls. To the first row of tubes was added equal volumes of saline, and to the succeeding rows equal volumes of extract as indicated in the tables.

The results were recorded as follows:

C	= one to three large clumps
+++	= smaller macroscopical clumps
++	= microscopical, mainly large clumps
+	= clumps of a dozen or more cells with many loose ones
w	= clumps of two or three cells
—	= no agglutination

Complement Fixation Tests.—These tests were based on the method described by Boyd (1947). The following scheme was adopted:

Extract emulsion	..	..	..	..	0.1 ml.
Serum	..	..	..	..	0.05 "
G.P. complement (2½ units)	..	..	..	..	0.25 "
Saline to make total volume of	..	..	..	..	0.7 "

The tubes were allowed to stand at 2° C. for 18 hours and then for 10 minutes at 37° C. after which 0.3 ml. of haemolytic system (2 vols. of haemolytic serum containing 5 units to 1 vol. of 5% suspension of washed sheep cells) was added. After incubating for 30 minutes at 37° C. the tubes were centrifuged at 1,500 r.p.m. for 2 minutes and readings taken. Results were recorded as follows:

C	= complete haemolysis
ac	= almost complete haemolysis
+	= partial haemolysis
Tr	= trace of haemolysis
—	= no haemolysis

Results

The results are divided into two parts, the first recording Carter's preparation, whilst the second comprises a few further experiments which seemed relevant to the problem.

Part I.—Not surprisingly, the extracts were found to be haemolytic as indicated in Table I, and this placed limitations on the concentrations which could be used.

Inhibition tests were carried out with anti-D sera, using extracts in minimal non-haemolytic dilutions, and typical results are shown in Tables II and III.

TABLE I
HAEMOLYTIC* TITRES OF EXTRACTS IN SALINE

Extract	Temperature	Dilutions in Saline					Control
		2	4	8	16	32	
"Positive" ..	Room (37° C.)	C	—	—	—	—	—
"Negative" ..	Room (37° C.)	C	C	—	—	—	—
		C	+	—	—	—	—
		C	C	+	—	—	—

* Haemolysis of D-positive red cells.

TABLE II
INHIBITION TEST WITH "POSITIVE" EXTRACT AT ROOM TEMPERATURE*

Row	Dilutions of anti-D in Saline									Controls
	2	4	8	16	32	64	128	256	512	
1. Saline ..	C	C	C	†	+++	+	—	—	—	—
2. Alcohol 1:4	C	C	C	+++	+++	w	—	—	—	—
3. Extract 1:4	C	C	+++	+++	+++	+	—	—	—	—

* Agglutination of D-positive red cells. † Spoilt tube.

TABLE III
INHIBITION TEST WITH "NEGATIVE" EXTRACT AT ROOM TEMPERATURE*

Row	Dilutions of Anti-D in Saline									Controls
	2	4	8	16	32	64	128	256	512	
1. Saline ..	C	C	+++	+++	+++	+++	+	—	—	—
2. Alcohol 1:4	C	C	+++	+++	+++	+	w	—	—	—
3. Extract 1:5	C	C	+++	+++	+++	+	—	—	—	—

* Agglutination of D-positive red cells.

It will be seen that in row 3 of Table II there is a slight loosening of the clumps, but there is no unequivocal inhibition, specific or otherwise.

In order to test higher concentrations of the extracts, dilutions of anti-D were set up in normal group AB serum, which inhibits the lytic effect. The result of such a test is shown in Table IV. It will be seen that there is relative inhibition by the "positive" extract, but it is of doubtful significance.

TABLE IV
INHIBITION TEST IN AB SERUM AT ROOM TEMPERATURE*

Row	Dilutions of Anti-D in AB Serum							Controls
	2	4	8	16	32	64	128	
1. "Positive" extract	+++	+++	+++	+	+++	—	—	—
2. "Negative" extract	+++	+++	+++	+++	+++	—	—	—

* Agglutination of D-positive red cells.

Finally, dried extracts were tried: 0.05 ml. of the 1% alcoholic solution was evaporated to dryness in a tube with a vacuum pump. Then 0.05 ml. of anti-D serum was added and the mixture allowed to stand at room temperature for one hour. The serum was run out in serial dilution and titrated. Table V shows the comparison of the results obtained simultaneously with "positive" and "negative" extracts. Again, there is no significant difference.

TABLE V
INHIBITION TESTS WITH DRIED EXTRACTS AT ROOM TEMPERATURE*

Row	Dilutions in Saline										Controls
	1	2	4	8	16	32	64	128	256	512	
1. Anti-D† alone ..		+++	+++	+++	+++	+++	+++	w	—		—
2. Anti-D+ positive extract ..	C	C	C	C	+++	+++	+	w	—	—	—
3. Anti-D+ negative extract ..	C	C	C	+++	+++	+++	+++	—	—	—	—

* Agglutination of D-positive red cells.

† An independent titration of the same serum included for comparison of the final titre.

Complement Fixation.—It was found that the extracts were anticomplementary, a not uncommon feature of tissue extracts, and attempts to determine anticomplementary titres at low temperature gave inconsistent results. In a series of 20 titrations on the same extract, results were obtained varying from 1 : 500 to 1 : 64,000. The widest variations appeared to be associated with different pools of guinea-pig serum used for complement and were independent of the age of the complement, as is shown in Table VI, in which different complement pools are compared. The guinea-pig serum was stored at -20°C . when not in use.

TABLE VI
ANTICOMPLEMENTARY TITRES USING DIFFERENT COMPLEMENT POOLS INCUBATED AT 2°C . OVERNIGHT*

Complement Pool (2½ units)	Dilutions of Extract (in hundreds)										Complement Controls
	5	10	20	40	80	160	320	640	1,280	2,560	
No. 1 (fresh) ..	Tr	+	ac	ac	C	C	C	C	C	C	C
No. 1 (17 days)†	ac	ac	ac	C	ac‡	C	C	C	C	C	C
No. 2 (10 days)	—	—	—	Tr	Tr	+	C	C	C	C	C
No. 3 (6 days)	+	+	ac	C	C	C	C	C	C	C	C
No. 4 (fresh) ..	—	—	—	—	Tr	+	ac	C	C	C	C
Preserved§ ..	—	—	—	—	—	—	—	+	ac	C	C

* Haemolysis of sensitized sheep red cells. † Complement stored at -20°C . ‡ Probable impurity in tube.
§ "Wellcome" preserved complement (Richardson's method).

Using Pool No. 3, which gave the lowest anticomplementary titre, complement fixation tests were performed using the positive extract and an anti-D serum having a saline agglutinin titre of 1 : 128. The result was negative, as shown in Table VII.

TABLE VII
COMPLEMENT FIXATION TESTS WITH "POSITIVE" EXTRACT AND ANTI-D SERUM INCUBATED
AT 2° C. OVERNIGHT*

Extract Dilution	Extract Controls		Dilutions of Serum					
	Haemo- lytic	Anti- complementary	Anti-D				Normal Serum	
			4	8	16	32	4	8
2,000	—	+	ac	+	+	+	ac	ac
4,000	—	ac	C	ac	ac	ac	C	C
8,000	—	C	C	C	C	C	C	C
16,000	—	C	C	C	C	C	C	C
32,000	—	C	C	C	C	C	C	C
Serum controls	Anticomplementary		C	C	C	C	C	C
	Haemolytic		—	—	—	—	—	—

* Haemolysis of sensitized sheep red cells.

Part II.—The conclusion to be drawn from these results is in harmony with the statements made by Coombs and Stratton.

In the preliminary tests, however, following the normal procedure with Rh antibodies, the inhibition tests were carried out at 37° C., and many irregular and inconsistent results were obtained in the form of haemolysis and partial inhibition. These appeared to be due to the effect of the alcohol on the serum.

When alcohol was incubated with normal serum at 37° C. for periods greater than one hour, the product became increasingly haemolytic, producing complete haemolysis at the end of two and a half hours' incubation. The effect was readily produced with 50% alcohol, and lower concentrations were also effective, but the effect of alcohol on different anti-D sera was very variable.

As no lysis occurred at room temperature the inhibition tests recorded were carried out at room temperature. It seemed probable that as a preliminary to lysis there was some loosening of the clumps which might simulate inhibition, and the variability of the effect might well give false impressions. However, the results obtained at room temperature were at least consistent.

Extracts with organic solvents inevitably contain much lipid material, and this group of substances is known to give rise to non-specific effects. Spielmann (1949) showed that purified ovo-lecithin would inhibit antibodies of the Rh system just like his purified alcoholic extracts of red cells, but would not apparently inhibit anti-A or anti-B.

Table VIII shows the results of inhibition tests with commercial ovo-lecithin and anti-sera of the ABO and Rh systems. A 1% solution of the ovo-lecithin in ethyl alcohol was made and diluted with saline to 1 : 40 (the lowest non-haemolytic dilution).

The preparation, which was used as received from the manufacturers without further purification, gave results somewhat at variance with those recorded by Spielmann. Inhibition is definite with the anti-A and anti-B, but slight with only one of the anti-D sera tested. The explanation may lie in the fact that Spielmann

TABLE VIII
INHIBITION TESTS WITH COMMERCIAL OVO-LECITHIN PERFORMED AT ROOM TEMPERATURE*

Serum	Equal Vols. of	Dilutions of Sera										Controls
		1	2	4	8	16	32	64	128	256	512	
Anti-A	Saline Lecithin (1:4,000)	C	C	C	C	C	+++	+	w	—	—	—
Anti-B	Saline Lecithin (1:4,000)	C	C	C	C	+	—	—	—	—	—	—
Anti-D	Saline Lecithin (1:4,000)	C	C	C	C	+++	+++	+++	+	—	—	—
Anti-D	Alcohol (1:40) Lecithin (1:4,000)			+++	+++	+++	+++	+	—	—	—	—
Anti-D	Saline Lecithin (1:4,000)		+++	+++	w	—	—	—	—	—	—	—
			+++	+++	—	—	—	—	—	—	—	—

* Agglutination of A, B, or D-positive red cells.

used a purer form of a substance which, in any case, appears to be unstable and deteriorates rapidly. In this connexion it should be mentioned that the first extract obtained from D-positive cells did produce a marked inhibition of one anti-D serum only (Table IX). It could never be repeated with other sera, and unfortunately that particular serum was no longer available later to be tested with a "negative" extract.

TABLE IX
INHIBITION TEST WITH "POSITIVE" EXTRACT AND AN ANTI-D SERUM PERFORMED AT 37° C.

Row	Dilutions of Anti-D										Control
	4	8	16	32	64	128	256	512	1024	2048	
1. Saline ..	+++	+++	+++	+++	+++	+++	+	w	—	—	—
2. 50% alcohol	+++	+++	+++	+++	+++	w	+	+	—	—	—
3. Extract ..	++	—	—	—	—	—	—	—	—	—	—

With regard to complement fixation it was thought possible that the actual technique of preparing the saline emulsion might influence the anticomplementary effect. However, a comparison of different methods of preparation (including allowing it to stand for two hours before use) revealed no difference. It was concluded, therefore, that the cause of the variation in anticomplementary effect of the extract was inherent in the material itself, especially since stabilized antigens do not give such remarkable variations in anticomplementary titres.

In view of the work of Landsteiner and van der Scheer (1925) on flocculation tests with alcoholic extracts, it was thought pertinent to investigate the activity of such extracts with anti-D sera. Extracts were prepared according to the original method of these workers, and the results are recorded in Table X. A dilution of 1:3 in saline was non-haemolytic. The result is seen not to be really significant, although there is a slight bias in favour of the "negative" extract.

TABLE X
INHIBITION TESTS WITH ALCOHOLIC EXTRACTS AND ANTI-D PERFORMED AT ROOM TEMPERATURE

Row	Dilutions of Anti-D								Controls
	2	4	8	16	32	64	128	256	
1. Saline	+++	+++	+++	+++	+++	+++	w	—	—
2. Alcohol 1:3 ..	+++	+++	+++	+++	+++	+	—	—	—
3. "Negative" extract 1:3 ..	+++	+++	+++	+++	+	w	—	—	—
4. "Positive" extract 1:3 ..	+++	+++	+++	+++	+++	w	—	—	—

Discussion

The well-recognized non-specific effects of lipid substances clearly render the use of a "negative" control vitally essential, but even without this the results of inhibition tests in this work are mainly without significance. Moreover, the difficulties of working with alcoholic solutions seem to make the whole method very uncertain.

In complement fixation we are dealing with a much more complicated and much less understood mechanism, and it seems doubtful whether it possesses any advantages over inhibition tests with regard to sensitivity. We do not know whether or in what circumstances the Rh system is capable of fixing complement. During the present investigation attempts to demonstrate complement fixation with whole D-positive cells and an anti-D serum at 37° C. ended in failure. It probably resembles the ABO system which Schiff and Adelsberger (1924) showed to fix complement very poorly if at all, although Ervin and Young (1950) claim to have obtained complement fixation with an immune anti-A.

In the present investigation, the prime difficulty in complement fixation was the anticomplementary effect of the extract. Even with a suitable complement, the usable dilution of extract (1:4,000) was probably high enough for any specific activity to be lost. The uncertain behaviour of the extract with complement demands great caution in the interpretation of such tests, and the possibility of summation of anticomplementary effects of antigen and antibody (Eagle, 1932) must also be taken into account.

That red cell extracts can display inhibitory properties cannot be denied, but they are not necessarily specific in character. Spielmann's more dramatic results with alcoholic extracts are presumably related to the longer period of extraction (eight days as opposed to 24 hours in the method used here), and it is significant that his purified preparation was less active than the crude product.

It is conceivable that the presence of lipid material in the extract may inhibit or otherwise mask its specific activity in *in vitro* tests. Spielmann produced a titre of antibodies in guinea-pigs by injecting group O, D-positive cells and demonstrated a fall in titre following the injection of his purified preparation of lecithin. The fall in titre of Rh antibody in two pregnant women observed by Carter need not be specific in the light of Spielmann's experiment, and it would be interesting to know

what effect the injection of lecithin would have on the titres of various other antibodies.

The experiments of other workers and those presented here confirm that lipid substances give rise to considerable difficulties in serological work. In particular, lecithin has given very variable results in the hands of different workers. A similar state of affairs appears to exist in respect of the present problem, as is shown by the work of Spielmann and of the present author. Until these discrepancies can be satisfactorily controlled, it seems premature to talk of the Rh hapten.

Summary

Carter's (1949) method for the extraction of the so-called hapten from Rh-positive red cells has been carefully followed, and the products obtained tested for activity and Rh specificity by means of inhibition and complement fixation tests.

No significant inhibition with the final ether extract, specific or otherwise, could be demonstrated with the majority of anti-D sera used.

Haemolytic and inhibitory effects of alcohol are discussed in relation to the difficulties of performing such tests with alcoholic solutions.

Inhibition tests were also carried out with alcoholic extracts of D-positive and D-negative cells, and again no significant inhibition could be demonstrated.

Commercial ovo-lecithin was shown to have inhibitory properties with anti-sera of both the Rh and ABO systems.

Complement fixation tests were carried out with D-positive cell extracts at 2° C. overnight. No fixation could be demonstrated. The chief difficulty with these tests was the anticomplementary effect of the extract, which showed marked variation according to the complement used.

It is emphasized that lipid substances are prone to give non-specific reactions and that the isolation of the Rh hapten has not been established on the published evidence.

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