THE INACTIVATION OF THE BLOOD GROUP RECEPTORS ON THE HUMAN ERYTHROCYTE SURFACE BY THE PERIODATE ION.

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THE rapidity with which dilute solutions of sodium periodate inactivate the water-soluble human blood group mucoids (Morgan, 1947*a* ; Aminoff and Morgan, 1949, 1951 ; Annison and Morgan, unpublished work) suggested that the examination of the action of this reagent on the intact human erythrocyte might yield some interesting information as to the relative susceptibility of the group receptors present, and perhaps give some indication of the nature of those blood group factors which do not normally appear in water-soluble form—for example, the materials associated with M, N, P and Rh specificity.

The use of periodate in virus receptor studies has demonstrated that treatment of red cells with the reagent does not result in extensive morphological damage to the cells. Thus Hirst (1948) examined the influence of a number of oxidizing agents on fowl red cells, and reported that, although sodium periodate in 0·001 M concentration rapidly abolished virus receptor activity and led to changes in haemoglobin colour, the morphology of the erythrocytes was unchanged.

An account of the action of potassium periodate on the human red cell was given by Stewart (1949), who showed that the specificity of the cell was changed in such a way that the treated cells were capable of stimulating the production of specific antibodies in the rabbit which did not react with untreated cells. Stewart also found that the erythrocytes after treatment with periodate were rendered panagglutinable by normal human sera. The behaviour of the specific blood group characters of the cells, however, was not examined. Moskowitz and Treffers (1950) confirmed the presence of an agglutinin in normal sera for red cells which had been treated with periodate, and reported that the A and B blood group receptors were not destroyed by treatment with 0·001 M potassium periodate at pH 7 for 30 minutes at room temperature. Under these conditions the *Rhesus* character (as measured by the D antigen) was apparently destroyed completely, but no details of the serological tests were given.

The results of a systematic examination of the action of periodate ions, at different concentrations and pH values and for different times, on the serological behaviour of the human blood group characters A, B, O, H, Le^a, *Rhesus* (D), M, N and P are reported in this paper.

EXPERIMENTAL.

Materials and Methods.

Periodate solutions.

Dilutions in normal saline were made from a stock 0·5 M-HIO_4 solution.

Buffer solutions.

Acetate buffer (M) was used for the range pH 4·0–5·0. For pH 7·0 Na_2HPO_4-KH_2PO_4 buffer (0·66 M) was used.

Treatment of red cells with periodate.

Red cells were treated with 0·005 M and 0·001 M periodate at pH 7·0, 5·0, 4·5 and 4·0. Freshly drawn citrated blood was centrifuged and the cells washed several times with normal saline. Suspensions (10 per cent) in saline were prepared from the packed cells, and 2 volumes were mixed with 1 volume of buffer solution and 1 volume of periodate solution. The cell suspensions were kept at 15 ± 1° with frequent but gentle mixing. Samples were withdrawn at intervals, diluted immediately with an equal volume of glucose-citrate solution (30 g. sodium citrate, 25 g. glucose to 1000 ml. water) in order to prevent further reaction of the periodate, centrifuged, and the deposited cells washed once with glucose-citrate solution. For use in the agglutination tests 1 per cent suspensions in glucose-citrate solution were prepared from the washed cells, and were then filtered through plugs of cotton-wool to remove any clumps of cells. Treatment with periodate at pH 4·0, 4·5 and 5·0 caused the cells to become brown in colour, but they appeared normal in shape, or at most only slightly distorted, and the suspensions were free from haemolysis. The rapidity with which the cells changed colour increased with increase in the acidity of the buffer and with the strength of periodate used. Red cells suspended in buffer solutions at these pH values, in the absence of periodate, did not change colour, and after centrifugation and washing gave suspensions in glucose-citrate solution which showed normal stability over a period of several hours. Treatment of red cells with 0·001 M periodate at pH 7·0 up to a period of 2 hours did not give rise to visible change in the cells, but exposure to 0·005 M periodate at this pH resulted in extensive haemolysis; red cells treated under these conditions could not therefore be tested for loss of blood group activity.

Test sera.

Human sera were used for A, B, O, P, Le^a and *Rhesus* (D) tests (Schiff and Boyd, 1942; Wiener, 1943; Race and Sanger, 1950). Owing to the presence of an agglutinin in all human sera for cells treated with periodate (Stewart, 1949; Moskowitz and Treffers, 1950) it was necessary to absorb these sera before use with periodate-treated cells which did not contain the blood group receptor under investigation. For example, in order to test the activity of A cells treated with periodate the anti-A serum used was previously absorbed with O cells treated with periodate. Sera were absorbed for 20 minutes at 10° with 0·1 volume of red cells which had been exposed to 0·005 M periodate at pH 5·0 for 30 minutes. The absorption was repeated until there was no appreciable agglutination of the absorbing cells by the undiluted serum. The H, M and N activities of cells were tested by means of immune rabbit sera. These sera did not contain agglutinins for cells treated with periodate and therefore absorption was unnecessary.

Agglutination tests.

The method consisted in each instance of adding a constant volume of cell suspension (1 per cent) to serial dilutions of serum, and leaving the mixture to

stand 2 hours at the appropriate temperature, after which time the tubes were examined for agglutination. The agglutination end-point was taken as the last dilution at which clumps of two or three cells were visible under the low power of the microscope. Differences in the strength of agglutination, the amount of serum available and the optimum temperature for the reaction necessitated slight variations in the technique employed in testing for the different factors. A and B tests were carried out by mixing 0·1 ml. of cell suspension, accurately measured, with an equal volume of serum dilution in tubes measuring 0·5 in. by 2 in. Owing to the limited amount of anti-M, N, H, O, Le^a, P and *Rhesus* (D) sera available, when tests were carried out with these reagents the serum dilutions and cell suspensions were measured into small tubes (0·25 in. by 2 in.) by means of a Pasteur pipette graduated at 0·02 ml. Throughout the reaction period A, B, M and N tests were kept at room temperature, H, O, Le^a and P at 12–14°, and *Rhesus* (D) cells were incubated with the serum at 37°. The tubes containing A, B, M and N cells and the corresponding agglutinins were given three standard taps before reading, whereas, in view of the ease with which the agglutinated cell clumps are broken up, H, O, Le^a, P and *Rhesus* (D) serum-erythrocyte suspensions were transferred gently and without shaking to the microscope slide and spread for observation as a thin film.

Absorption tests.

In addition to agglutination tests with the red cells after treatment with periodate, the destruction of the specific blood group receptors was measured in some instances by following the decrease in the capacity of the cells to absorb antibody from the homologous serum. The volume of packed untreated cells of the appropriate group which it is necessary to add in order to absorb the specific antibody almost completely from a given volume of serum was determined for each serological character in a preliminary experiment. Suspensions (10 per cent) in glucose-citrate solution were prepared from red cells treated with periodate as described above, and a sample containing the previously determined volume of cells was measured into a centrifuge tube. The suspension was centrifuged, the supernatant removed and the given amount of serum was added to the deposited cells. The mixture was stirred with a glass rod to ensure an even suspension, and was then kept at 8–10° for 30 minutes with frequent shaking. The agglutination titre of the absorbed serum with untreated cells of the appropriate group was then determined.

Precipitation tests.

The action of periodate on protein solutions was followed by means of serological precipitation tests. These tests were made by mixing 0·1 ml. of undiluted test-serum with 0·1 ml. of serial dilutions of the treated protein solution in normal saline. After 2 hours at 37° the tubes were kept overnight at 0° and examined while still cold by means of a hand lens.

Treatment of protein solutions with periodate.

Protein solutions diluted 1 : 500 (2 ml.) were added to 0·02 M HIO_4 solution (1 ml.) and buffer solution pH 5·0 (1 ml.) and the mixtures were allowed to stand at 15°. Samples were removed after 15, 45 and 120 minutes and diluted with

an equal volume of 0·05 M-Na_2HPO_4 in glucose-citrate solution. Addition of this latter solution served to bring the pH to 7 and to remove excess periodate. The solutions were then centrifuged and tested for precipitation with the homologous antiserum.

Coombs test.

The indirect test described by Coombs, Mourant and Race (1945) for the detection of " non-agglutinating " *Rhesus* antibodies was used in order to obtain red cells with a surface coating of protein which could be detected by means of agglutination tests. Human group O homozygous *Rhesus* positive (DD) cells (20 per cent) suspended in normal saline were added to an equal volume of " non-agglutinating " anti-D serum, and the mixture was incubated at 37° for 30 minutes. The suspension was then centrifuged and the cells were washed twice with saline. A 1 per cent suspension in saline was prepared from the washed cells and was added to serial dilutions of rabbit anti-human-γ-globulin serum. Agglutination of the treated cells was thus an indication that combination with the " non-agglutinating " antibody (γ-globulin) had occurred. *Rhesus*-negative cells of group O which had been similarly treated with " non-agglutinating " anti-D serum, and *Rhesus*-positive (DD) of the same group cells which had not undergone previous treatment, were included as controls in the agglutination tests with the anti-γ-globulin serum.

Preparation of stromatin.

Stromatin from human red cells was prepared by the method of Calvin, Evans, Behrendt and Calvin (1946). Group A cells were washed twice with saline, cooled to 0°, and then haemolysed by pouring into 10 volumes of water at 0–4°. N/1 HCl was cautiously added with thorough stirring until the pH was reduced to 5·7. The precipitated stromata were rapidly collected in a Sharples centrifuge, and were then washed alternately with dilute phosphate buffer pH 5·0 and distilled water until the supernatant was free from haemoglobin. The stromata were resuspended at pH 7·6, and centrifuged at 50,000 G. Stromatin was obtained from the clear supernatant fluid by readjustment of the pH to 5 with dilute acetic acid. The precipitate was centrifuged, suspended in saline at 0°, and the pH brought to about 7·3 with dilute alkali. The resultant clear solution was stored at 2–3° in the presence of 1 : 1000 merthiolate.

Preparation of anti-stromatin serum.

Three rabbits were given 4 intravenous injections of stromatin (1 mg., 2 mg., 5 mg., 5 mg.) spaced at 2–3 day intervals. Serum samples, taken 8 days after the last injection, gave precipitates with the injected antigen.

RESULTS.

(1) *The Influence of Periodate on the Specific Blood Group Receptors on the Erythrocyte Surface.*

Treatment of human erythrocytes with periodate resulted in loss of activity of all the blood group factors examined, but it was possible by varying the concentration of periodate used and the pH at which the reaction was carried out to

detect differences in the relative susceptibilities of the various specific receptors to the action of the oxidizing agent.

(a) *The A_1 and B receptors.*

The results of a typical inactivation experiment showing the fall in A specific activity after exposure of A_1 cells to two concentrations of periodate for various time intervals, and at different pH values, are given in Table I. Group O cells

TABLE I.—*Inactivation of A Structures on the Erythrocyte Surface by Periodate.*

Cell group.	Treatment. pH.	IO_4' (M conc.).	Time (min.).	Dilution of anti-A test serum. 1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.	1 : 256.
A	4·0	—	15	3	3	3	3	2	1	tr.
,,	,,	0·001	,,	1	tr.	0	0	0	0	0
,,	,,	0·005	,,	tr.	0	0	0	0	0	0
O	,,	,,	,,	tr.	0	0	0	0	0	0
A	4·5	—	15	3	3	3	3	2	1	tr.
,,	,,	0·001	,,	2	2	1	tr.	0	0	0
,,	,,	0·005	,,	1	tr.	0	0	0	0	0
O	,,	,,	,,	tr.	0	0	0	0	0	0
A	5·0	—	120	3	3	3	3	2	1	0
,,	,,	0·001	15	3	3	3	3	2	1	0
,,	,,	,,	45	3	3	3	2	1	tr.	0
,,	,,	,,	120	3	2	2	1	0	0	0
O	,,	,,	,,	1	0	0	0	0	0	0
A	,,	0·005	15	3	3	3	2	1	0	0
,,	,,	,,	45	2	1	0	0	0	0	0
,,	,,	,,	120	1	tr.	0	0	0	0	0
O	,,	,,	,,	tr.	0	0	0	0	0	0
A	7·0	—	,,	3	3	3	3	2	1	tr.
,,	,,	0·001	15	3	3	3	2	2	1	tr.
,,	,,	,,	45	3	3	3	2	2	1	0
,,	,,	,,	120	3	3	3	2	2	1	0
O	,,	,,	,,	1	0	0	0	0	0	0

Degrees of agglutination : tr., trace of agglutination ; 1, groups of 2–3 cells ; 2, larger groups with many free cells ; 3, agglutination visible to the naked eye.

were also treated with periodate under the appropriate conditions and included in each test to measure the amount of agglutinin remaining in the absorbed test-serum for cells treated with periodate. Under the most drastic conditions used (0·005 M HIO_4 at pH 4·0) the agglutination end-point with A_1 cells fell from 1 : 256 to 1 : 4 after exposure of the cells for 15 minutes, thus indicating that approximately 1 per cent only of the original activity remained. Treatment with more dilute periodate (0·001 M) at this pH again resulted in extensive inactivation, and a reduction of five tubes in the agglutination end-point was observed. At pH 5·0 the more dilute periodate was less effective, and treatment for two hours was necessary before a change measurable by the agglutination technique could be detected, but with 0·005 M periodate the activity had fallen to approximately 50 per cent of the original in 15 minutes and to about 6 per cent after 45 minutes. The fall of one tube in the agglutination end-point after exposure of the cells to 0·001 M periodate at pH 7·0 for 45 minutes was not increased after treatment for 2 hours, and this result suggests that very little inactivation of A receptors on the erythrocyte surface occurs in the presence of dilute periodate in neutral

solution. The course of inactivation of the B factor was essentially similar to that described for A ; the results are summarized in Table II.

In order to verify that the fall in the agglutination end-point observed after treatment of A_1 cells with periodate was due to inactivation of the specific A

TABLE II.—*Inactivation of A, B, H and O Structures on the Erythrocyte Surface by Periodate.*

Treatment of red cells.			Agglutination titre of cells with homologous antiserum.			
pH.	IO_4' (M conc.).	Time (min.).	A.	B.	O.	H.
4·0	—	15	256	512	256	1280
,,	0·001	,,	8	16	4	80
,,	0·005	,,	4	8	4	40
4·5	—	,,	256	256	—	—
,,	0·001	,,	32	32	—	—
,,	0·005	,,	8	8	—	—
5·0	—	120	128	512	256	2560
,,	0·001	15	128	512	256	2560
,,	,,	45	128	256	128	1280
,,	,,	120	32	128	16	320
,,	0·005	15	64	256	32	1280
,,	,,	45	8	8	8	160
,,	,,	120	8	4	4	80
7·0	—	,,	256	512	256	2560
,,	0·001	15	256	512	256	1280
,,	,,	45	128	256	128	640
,,	,,	120	128	256	64	640
Control cells after treatment with IO_4'			O	4	4	—	—
			A_1B	—	—	4	40

receptors, and not to other changes in the red cells which prevented agglutination taking place, absorption experiments were carried out with the cells after treatment with periodate. Thus, if the A_1 receptors remained unchanged and combined with anti-A agglutinins, although agglutination failed to occur, the serum absorbed with the periodate-treated cells would show the same lowering of the agglutination end-point as would serum absorbed with untreated A_1 cells. The results of such an experiment given in Table III show that the absorptive power

TABLE III.—*The Agglutination Titre of Anti-A Serum with Untreated A_1 Cells after Absorption of the Serum with A_1 Cells Treated with Periodate.*

Cells used for absorption.				Agglutination titre of absorbed serum against untreated A_1 cells.
Group.	Treatment.			
	pH.	IO_4' (M conc.).	Time (min.).	
A	—	—	—	4
,,	5·0	0·005	15	4
,,	,,	,,	45	64
,,	,,	,,	120	64
O	,,	,,	,,	512
A	7·0	0·001	15	4
,,	,,	,,	45	4
,,	,,	,,	120	4
Titre of unabsorbed serum with A_1 cells				1024

of the A_1 cells diminishes after treatment with 0·005 M periodate at pH 5·0 for periods up to 2 hours, but exposure to 0·001 M periodate at pH 7·0 for this length of time did not give rise to a detectable decrease in the absorbing capacity of the cells. These results, therefore, confirm the findings obtained in simple agglutination tests with A_1 cells treated with periodate.

(b) *The O and H receptors.*

The differentiation of so-called anti-O sera into anti-O and anti-H, and the recognition of two distinct receptors, designated O and H, on the red-cell surface, are based on the reactions of a human anti-O serum examined by Boorman, Dodd and Gilbey (1948). These authors investigated the behaviour of 500 blood samples with this serum, and found that the results were in very close agreement with the frequencies which would be expected if the antibody were reacting with the product of the Bernstein O (and A_2) gene. A comparison of the reactivity of this serum with cells of different genotypes with the reactivity of the so-called anti-O sera from cattle, from goats immunized with *Sh. shigae* and from rabbits injected with purified " O-substance " prepared from human pseudo-mucinous ovarian cyst fluids (Morgan and Waddell, 1945), revealed discrepancies which could not be explained if the latter group of sera was also detecting the product of the O gene. It was proposed, therefore, that the factor detected by these sera should be called H, and that the symbol O should be reserved for the product of the Bernstein O gene ; the corresponding antibodies were therefore termed anti-H and anti-O (Boorman, Dodd and Gilbey, 1948 ; Morgan and Watkins, 1948). Examination of a number of human sera which agglutinate O cells has since revealed several examples of sera which behave similarly to the anti-O serum described by Boorman, Dodd and Gilbey (1948). In addition human sera are not infrequently encountered which are similar in reactivity to the animal anti-H sera.

The influence of periodate on the O and H receptors present in group O cells is shown in Table II, in which the agglutination titres with the homologous antisera before and after treatment of the cells with the oxidizing agent are recorded. On the whole, loss of O specific activity was slightly more rapid than loss of H activity, but the difference was not very marked. After treatment for 15 minutes with 0·001 M periodate at pH 4·0 a lowering of six tubes in the agglutination end-point of the treated cells with anti-O serum, and a reduction in the end-point of four tubes with anti-H serum, was observed. Exposure to 0·005 M periodate at this pH resulted in almost complete inactivation of both factors. At a rather less acid pH (5·0) both O and H receptors were relatively unchanged by treatment with 0·001 M periodate for periods up to 45 minutes, but after 2 hours the H activity fell to about 12 per cent and the O activity to 6 per cent of the original value. The greatest difference in the susceptibilities of the two factors was apparent after treatment with 0·005 M periodate at pH 5·0 for 15 minutes, when the agglutination end-point with anti-H serum was lowered by only 1 tube (about 50 per cent of original activity remaining), whereas the end-point with anti-O serum fell by four tubes (about 12 per cent of the original activity remaining). The O and H specific structures both appeared to be fairly resistant to the action of 0·001 M periodate at pH 7·0, and treatment of O cells for 2 hours resulted in a reduction of the agglutination end-point of two tubes only with both anti-O and anti-H sera.

(c) *The M and N receptors.*

The M and N receptors were found to be more susceptible to the action of periodate ions than any of the other factors which have been so far considered. The agglutination titres of M and N cells, belonging to group O, with the corresponding antisera after treatment of the cells with 0·001 M periodate at pH 5·0 and 7·0 are recorded in Table IV. After contact with the oxidizing agent for 15 minutes

TABLE IV.—*Inactivation of M, N, P,* Rhesus *(D) and Le^a Structures on the Erythrocyte Surface by Periodate.*

Treatment of red cells.			Agglutination titre of cells with homologous antiserum.				
pH.	IO_4'. (M conc.).	Time. (min.).	M.	N.	P.	*Rhesus* (D).	Le^a.
4·0	—	15	—	—	32	—	32
„	0·001	„	—	—	4	—	8
„	0·005	„	—	—	2	—	4
5·0	—	120	32	64	64	512	64
„	0·001	15	4	16	64	64	64
„	„	45	2	8	64	16	64
„	„	120	2	8	16	4	16
„	0·005	15	—	—	64	16	32
„	„	45	—	—	32	4	8
„	„	120	—	—	16	4	8
7·0	—	„	32	64	64	512	64
„	0·001	15	4	16	64	64	64
„	„	45	2	8	64	32	64
„	„	120	2	8	32	16	32
Control cells after treatment with IO_4'	N		2	—	—	—	—
	M		—	8	—	—	—
	P-negative		—	—	4	—	—
	Rh-negative		—	—	—	4	—
	Le(a-)		—	—	—	—	2

at pH 5·0 group M cells retained about 6 per cent only (a reduction of 4 tubes in the agglutination end-point) of the activity of untreated M cells against anti-M serum, and after 2 hours at this pH inactivation was complete. At pH 7·0 the reaction took place more slowly, but a lowering in the end-point of two tubes was apparent after 15 minutes' treatment, and about 3 per cent only of the original reactivity remained after the periodate had been allowed to react with the cells for 2 hours. The anti-N test serum (titre 1 : 64), showed some reactivity with M cells (1 : 8) and it was possible therefore to detect only a 4-tube reduction in the agglutination end-point (12 per cent or less activity remaining). The results indicate, however, that inactivation of the N receptors was practically complete after 45 minutes' treatment with 0·001 M periodate at both pH 5·0 and 7·0.

The agglutination titres, obtained with untreated M cells, of samples of anti-M serum which had been absorbed under standard conditions with M cells treated with 0·001 M periodate for various lengths of time are shown in Table V. The failure of the cells to absorb significant amounts of antibody from the serum provides additional evidence that rapid loss of M specificity occurs in the presence of periodate ions.

TABLE V.—*The Agglutination Titre of Anti-M Serum with Untreated M Cells after Absorption of the Serum with M Cells Treated with Periodate.*

Cells used for absorption.				Agglutination titre of absorbed serum against untreated M cells.
Group.	Treatment.			
	pH.	IO_4' (M conc.).	Time (min.).	
M	5·0	—	120	2
„	„	0·001	15	32
„	„	„	45	32
„	„	„	120	32
N	„	„	„	32
M	7·0	—	„	2
„	„	0·001	15	16
„	„	„	45	32
„	„	„	120	32
N	„	„	„	32
Titre of unabsorbed serum with M cells				32

(d) *The P receptor.*

The results of the action of periodate on the agglutinability of P-positive cells belonging to group O with human anti-P serum are summarized in Table IV. At pH 4·0 the activity of the cells was reduced to about 12 per cent after treatment for 15 minutes with 0·001 M periodate, and to 6 per cent of the original value after treatment for the same length of time with 0·005 M periodate. The agglutination end-point of the cells was unchanged after treatment with the more dilute periodate (0·001 M) at pH 5·0 for 45 minutes, and had only fallen 2 tubes after the oxidizing agent had been allowed to react for 2 hours. In the presence of the higher concentration of periodate (0·005 M) at pH 5·0 the P-specific structures appeared to be more stable than the other blood group factors examined, and about 25 per cent of the original activity remained after 2 hours' treatment. In neutral solution 0·001 M periodate brought about a lowering in the agglutination end-point of 1 tube only after 2 hours, thus indicating that 50 per cent or more of the original activity remained unchanged. In all the tests described P-negative cells belonging to group O treated with periodate under the same conditions as the P-positive cells were included as controls.

Absorption experiments with P-positive cells which had been exposed to 0·001 M periodate at pH 7·0 and with cells treated with 0·005 M periodate at 5·0 confirmed the results obtained by means of the agglutination tests, and supported the conclusion that the P group receptor was one of the structures most resistant to the reagent.

(e) *The Le^a receptor.*

A summary of the results of the inactivation of Le^a structures on the erythrocyte surface by periodate is included in Table IV. The tests were carried out with group O, Le (a+) cells. Group O, Le (a—) cells treated under the appropriate conditions with periodate were also included in the agglutination tests as a measure of the residual agglutinin for cells treated with periodate remaining in the absorbed anti-Le^a serum. It will be observed that inactivation of the Le^a factor follows a course similar to that of the P receptors. The activity was reduced to about 25 per cent of the original value after treatment for 15 minutes

with 0·001 M periodate at pH 4·0, and to about 12 per cent with 0·005 M periodate. At pH 5·0 it was not possible to detect any loss of activity until after 2 hours' treatment with 0·001 M periodate, although more rapid inactivation occurred in the presence of more concentrated periodate (0·005 M). As had been found previously for A, B, H and P serological characters, loss of activity with 0·001 M periodate at pH 7·0 proceeded very slowly, and the agglutination end-point was lowered by only one tube after the reagent had been allowed to react with the cells for 2 hours.

(f) *The* Rhesus (*D*) *receptor.*

The influence of periodate on the *Rhesus* antigens on the erythrocyte surface was followed in the case of the D antigen by measuring the agglutination titre of the cells, before and after treatment, with an anti-D serum, and also by testing the capacity of the treated cells to absorb the antibody from anti-D serum. For the agglutination tests a potent anti-D serum (titre 1 : 512 in saline) was selected. Preliminary absorption experiments carried out with this serum, however, revealed that as many as five absorptions were necessary to cause a measurable lowering of the titre. This serum did not therefore appear to be suitable for absorption tests with the treated cells, and it was suggested to us by Dr. R. R. Race that the difficulty was due to the presence of considerable amounts of "non-agglutinating" D antibodies in the serum, which preferentially coated the cells and prevented their combination with, and the removal of, the antibody which acted in saline solution. In order to overcome this difficulty a number of less potent anti-D sera which agglutinated cells in saline were examined, and one was selected from which the antibody could be removed almost completely by one absorption with an equal volume of packed D cells.

The agglutination titres with anti-D serum of D cells treated with 0·001 M periodate at pH 5·0 and 7·0, and with 0·005 M periodate at 5·0, are given in Table IV. It is to be noted that inactivation of the D receptors occurred rapidly at both pH values. The agglutination end-point was lowered by five tubes after treatment of the cells for 45 minutes with 0·001 M periodate at pH 5·0, and with more concentrated periodate (0·005 M) for the same length of time at pH 5·0 the end-point fell to that given (1 : 4) by *Rhesus*-negative control cells which had been treated with periodate. It would therefore appear that under these conditions complete inactivation of the specific D structures occurs. At pH 7·0 treatment with 0·001 M periodate brought about a lowering of the agglutination end-point from 1 : 512 to 1 : 64 after 15 minutes and to 1 : 16 after 2 hours, thus indicating that about 3 per cent only of the original activity remained at the end of this time.

Absorption experiments with D cells treated with periodate confirmed the rapid inactivation of the D serological character which was observed when the treated cells were tested for agglutination. Absorption of the anti-D serum with untreated D cells reduced the agglutination end-point from 1 : 128 to 1 : 4, whereas after absorption with similar cells exposed to 0·001 M periodate at pH 7·0 for 15 minutes a one-tube reduction only in the end-point was obtained, and after more prolonged treatment with the oxidising agent the cells failed to remove sufficient antibody from the serum to cause any observable change in the agglutination titre.

(2) *The Influence of Periodate on Stromatin as Measured by the Specific Precipitation Reaction.*

The immunization of rabbits with stromatin was undertaken in the hope that agglutinins for a naturally occurring protein component of the human erythrocyte surface would thereby be produced. In the event of obtaining such antibodies it would thus be possible to investigate the influence of periodate on a component of the red cell membrane known to be protein in nature. The sera from the immunized rabbits reacted with the injected antigen in precipitation tests, but no increase in the agglutination titre was obtained with human red cells, other than those of group A from which the stromatin was prepared.

In order to determine whether the serological specificity of stromatin is destroyed by contact with periodate, a 0·1 per cent solution of the protein was treated at pH 5·0 with a 0·005 M solution of the oxidizing agent for various lengths of time, and was then tested for precipitation with rabbit anti-stromatin serum. The results showed that although there was a slight weakening in the degree of precipitation after treatment for 2 hours a considerable measure of the specificity of the native protein was retained.

(3) *Action of Periodate on Erythrocytes Sensitized with " Non-agglutinating " Antibody.*

In view of the failure to produce antibodies specific for a naturally occurring protein component of the erythrocyte surface, an attempt was made to attach protein molecules to the red cell by artificial means and to investigate the action of periodate on the attached protein by means of an antibody specific for the protein. The method used was based on the anti-globulin test described by Coombs, Mourant and Race (1945) for the detection of " non-agglutinating " *Rhesus* antibodies. Homozygous Rh-positive cells (DD) of group O sensitized with " non-agglutinating " anti-D serum were exposed to the action of 0·005 M periodate at pH 5 and 0·001 M periodate at pH 7 for various time intervals and were then tested for agglutination with anti-human-γ-globulin serum. Treatment with the more dilute periodate at pH 7 for 2 hours did not result in any lowering in the agglutination titre of the sensitized cells, but with 0·005 M periodate at pH 5·0 a seven-tube decrease in the end-point was observed after exposure of the cells to the reagent for only 15 minutes. Sensitized cells allowed to stand for a corresponding length of time in buffer solution at pH 5, in the absence of periodate, and subsequently washed twice with saline in the normal way, were agglutinated to the same extent by the anti-γ-globulin serum as were sensitized cells which had not been subjected to this treatment. The failure of the sensitized cells after treatment with periodate to react with the serum was therefore not due to the action of the acid buffer.

Precipitation reactions with anti-human-γ-globulin serum and solutions of purified human-γ-globulin (Kekwick and Mackay, 1949) (0·1 per cent), which had been allowed to react with 0·005 M periodate at pH 5 for various time intervals up to a total period of two hours, failed to reveal any loss of serological specificity.

DISCUSSION.

A study has been made of the action of sodium periodate under different conditions of pH and concentration on certain of the blood group receptors of

the human erythrocyte. The degree of inactivation has been measured by the fall in the capacity of the treated cells to react in simple agglutination tests with the appropriate serum and, in certain instances, by the power of the treated cells to absorb the specific antibody from the test serum.

The results obtained reveal that treatment of erythrocytes with periodate ultimately brings about the loss of all the specific serological characters examined. It is possible, however, by varying the concentration of periodate, time of reaction and pH, to demonstrate a differential inactivation of the group factors studied. For example, if the results obtained after treating the cells for 15 minutes with 0·001 M periodate at pH 5 are considered, it is evident that the specific group receptors can be divided into two categories—those susceptible to oxidation under these conditions and those resistant. To the former group belong the specific receptors M, N and *Rhesus* (D), and to the latter the structures specific for the gene products A, B, H, P, Le^a and O. A further differentiation of this latter group of receptors is brought about if 0·005 M periodate is used at pH 4 for 15 minutes. Under these conditions the A, B, H and O receptors are almost completely destroyed, whereas the P and Le^a structures are still detectable serologically. It is doubtful, however, whether the greater resistance of the P and Le^a receptors is sufficient to render this additional differentiation of practical importance. The results obtained at pH 7 confirm the observation of Moskowitz and Treffers (1950) that the A and B receptors are not appreciably inactivated by contact with 0·001 M periodate for 30 minutes, whereas the *Rhesus* (D) activity of the cells is largely destroyed.

The differences observed in the susceptibilities of the specific blood group structures to the action of periodate provide evidence that the failure of the cells to agglutinate with the specific blood group antibodies after exposure to the oxidizing agent is, in fact, due to inactivation or destruction of the specific receptors on the erythrocyte surface, and not due simply to other changes in the agglutinating properties of the cells ; if the latter were the explanation the rapidity with which loss of serological activity was observed would be expected to be constant irrespective of the blood group system under investigation. Further evidence that some abnormal state of the cells is not responsible for the lack of agglutination is provided by the fact that after treatment with periodate the cells are readily agglutinated by normal human sera (Stewart, 1949 ; Moskowitz and Treffers, 1950).

It is to be anticipated that the rate at which a susceptible blood group receptor is inactivated by periodate will be influenced not only by its chemical nature and structure, but also by the relative quantity and accessibility on the erythrocyte surface of the structures concerned. Nothing is known of the quantities of the various specific factors present, or of the spatial relationships of these determinant groups to other structures which compose the erythrocyte surface, and compared with the present knowledge of the chemistry of the water-soluble blood group substances, which have been identified as polysaccharide-amino acid complexes, relatively little is known concerning the chemical nature of the blood group receptors on the erythrocyte surface. The work of Hallauer (1934) and of Stepanov, Kuzin, Markageva and Kosjakov (1940) indicates that the A and B specific blood group materials obtained from erythrocytes are largely of a carbohydrate nature, and it seems reasonable to assume therefore that the water-soluble group substances (see Morgan, 1947*b* ; Kabat, 1949 ; Bray and Stacey, 1949) isolated

from tissue fluids and secretions constitute the specifically reactive materials on the erythrocyte surface. In consideration of the fact that the specific receptors on the cell surface are not removed, or are removed only with difficulty by repeated washing with saline, it may be assumed that these water-soluble group specific mucoids are combined with components of the cell membrane.

An investigation of the action of periodate on the specific blood receptors on the erythrocyte surface was undertaken in the hope that some insight would be gained into the nature of those group factors which do not occur to any appreciable extent in a water-soluble form, and which have not yet been identified chemically. Periodic acid differs from the common oxidizing agents in that it reacts mainly with compounds having two hydroxyl groups or a hydroxyl and an amino group attached to adjacent carbon atoms. This selectivity of action has led to a widespread application of the reagent for the determination of the constitution of carbohydrates. The complete inactivation of a specific blood group receptor on the erythrocyte surface, however, cannot be accepted as proof of its carbohydrate nature, for in addition to the reaction with α-glycol- and α-amino-alcohol groupings, oxidation of a number of amino-acids which do not contain these structures has been shown to occur (Jackson, 1944), although, admittedly, the reactions with these compounds proceed at a markedly slower rate. The nature of these reactions has not been established, but their existence makes it impossible to exclude substances from being protein in nature simply because their activity is lost on treatment with periodate.

Desnuelle and Antonin (1946) measured the formaldehyde liberated on treatment of a number of proteins with periodate, and found that the only protein that gave rise to a measurable quantity of formaldehyde was one which contained hydroxylysine, i.e., gelatin. It was therefore concluded that the reagent produced only minor changes in the proteins. Oxidative changes in the amino-acids which do not contain α-amino-alcohol groupings, however, fail to bring about the liberation of formaldehyde, and therefore would not be detected by this method. The observation that the toxicity of somatic antigens of *Shigella paradysenteriae*, which was believed to be associated with the protein component of the lipocarbohydrate protein complex, was diminished on treatment with periodic acid (Goebel, 1947), prompted this author to examine the action of the oxidizing agent on a number of biologically active compounds known to be protein in nature. The activity of crystalline ribonuclease against its specific substrate and of Type III pneumococcus immune globulin as measured by its power to precipitate with Type III polysaccharide were destroyed by treatment with the reagent, as was also the pathogenic action of the virus of Western equine encephalomyelitis (Goebel, Olitsky and Saenz, 1948). The loss of activity of the pneumococcus Type III immune globulin was also accompanied by changes in the absorption spectrum, but neither the species specificity of the protein nor the power to induce the formation of specific immune bodies was lost. In contrast the antigenicity of the equine virus was rapidly destroyed. A study of the physical, chemical and immunological changes in bovine serum albumin brought about by oxidation with lithium periodate (Goebel and Perlmann, 1949) revealed that destruction of certain amino-acids occurred, that the electrophoretic pattern was different after oxidation, that a change in the absorption spectrum was produced, and that prolonged treatment with lithium periodate destroyed the ability of the protein to induce the formation of antibodies. The treated albumin, however, was still

capable of precipitating some of the antibodies in sera of rabbits immunized with native serum albumin. It is therefore clear that, although the mechanism of the reactions involved has yet to be elucidated, significant changes in protein molecules can occur as a result of treatment with periodate ions. Nevertheless, for purposes of comparison with the results obtained for the blood group factors of unknown chemical composition, such as M, N, P and *Rhesus*, it appeared of interest to examine the action of the reagent on a surface component of the red cell known to be protein in nature. Attempts to do this, however, did not meet with success. Immunization of rabbits with a protein, stromatin, prepared from human erythrocyte stroma failed to elicit the formation of agglutinins for red cells, in spite of the fact that precipitins for stromatin were present in the serum. One might conclude from these observations that stromatin is not a serologically active component of the erythrocyte surface. The results obtained on treatment of *Rhesus* (D) cells sensitized with " non-agglutinating " antibody were inconclusive. The complete and rapid loss of activity of the sensitized cells against anti-γ-globulin serum after treatment with 0·005 M periodate at pH 5 is in sharp contrast to the results obtained with 0·001 M periodate at pH 7·0, where no inactivation was observed after 2 hours' treatment, and suggests changes occurring under the influence of the reagent other than oxidation of the attached γ-globulin. If the sensitized cells had not lost their capacity to agglutinate with anti-γ-globulin serum it would have been possible to state that the serological activity of the attached protein was unaffected by the oxidizing agent, but loss of activity cannot be accepted as proof that oxidative changes have occurred, in view of that fact that the bonds involved in antigen-antibody combination are labile and readily dissociated. Treatment of solutions of stromatin and γ-globulin with periodate under conditions of pH, time and concentration identical with those used for studying the action of the reagent on the red cell receptors did not result in loss of serological specificity as measured by their power to precipitate with the homologous antisera, but it need not be stressed that the rate of inactivation of a protein in solution is not necessarily comparable with the rate of inactivation of the same substance when it is present as a specifically orientated component of the red cell surface.

The results of the investigation do not allow a conclusion to be drawn as to the nature of those blood group factors which have not been identified chemically. It may be said, however, that those receptors whose specificity is almost certainly due to carbohydrate, A, B, H and Le^a, are inactivated more slowly by periodate than are the M, N and *Rhesus* D factors. Although a direct comparison between the rates of inactivation of the serological properties of carbohydrate and protein components of the erythrocyte surface has not been possible, it appears from the results of other experiments that proteins are on the whole less readily attacked than are carbohydrates. This being so, it will not be surprising if those receptors highly susceptible to treatment with periodate are subsequently found to be carbohydrate in nature.

SUMMARY.

(1) A study has been made of the action of periodate ions on a number of different specific blood group receptors on the human erythrocyte surface.

(2) The relative susceptibility of the receptors to the action of periodate was

measured by the loss of agglutinability with the homologous serum, and by the fall in the power of the treated erythrocytes to absorb the corresponding antibody from solution.

(3) The significance of the changes observed in the serological reactivity of the erythrocyte surface after exposure to periodate is discussed.

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