pregnancy. It would be necessary to assume that in the 4 false-negative cases cited in Table I, the concentration of blood gonadotrophin, when the serum was tested, was less than 2,500 I.U. per liter, despite the fact that it was 63 to 85 days after definitely established fertilization. The false-positive tests, *i.e.* positive reaction with serum of non-pregnant mares, occurred in 11-, 13- and 18-year-old animals. It is possible that age may increase the blood level of gonadotrophin in equids more markedly than in postmenopausal women, but it is unknown to what extent.

Since positive or negative responses are unreliable in 17% of the cases, it seems that the ovarian hyperemia test in immature rats can be used only as a first approach to the exact diagnosis of pregnancy in equids.

Summary. The ovarian hyperemia reaction in immature rats was studied following the subcutaneous injection of equine serum from 38 mares and 4 female donkeys, 42-138 days after natural insemination. The accuracy of this 24-hour pregnancy test was 83%, failures being observed both by negative reaction in 4 pregnant cases and positive reaction in 3 non-pregnant ones.

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Effect of Ozonized Oxygen on Anti-A, Anti-B, and Anti-Rh Typing Sera.*

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Reports of attempts to modify agglutinating and "blocking" antibodies against human erythrocytes have recently appeared in the literature. Boyd,[1] following the lead of Tyler,[2] reported on the effect of photo-oxidation on isohemagglutinins, anti-A and anti-Rh in the presence of Eosin Y as a sensitizer. Photo-oxidation produced by photoflood lights gradually destroyed the agglutinating activity but did not convert any of the agglutinins studied into "inhibiting," "incomplete," or "blocking" antibodies. An exposure of 15½ hours to these photoflood lights was sufficient to destroy completely the anti-Rh agglutinin. Anti-A agglutinin, however, required an exposure of 65 to 71 hours to destroy all agglutinating activity. Although it seems likely that the action of the photoflood lights in Boyd's experiments was that of oxidation, the possibility remains, nevertheless, that some other photo-dynamic effect may have played a part in modifying the agglutinins. It seemed desirable to us, therefore, to determine the effects on such agglutinins using a different oxidizing agent.

Materials and Methods. In this study ozone was employed to determine the effect of oxidation on isohemagglutinating antibodies, since this agent has useful attributes not possessed by other oxidizing agents. There are, for example, no added end products of the reactions other than those formed from the use of O_3. Ozone is a powerful oxidizing agent that will combine with unsaturated carbon atoms and form ozonides. The normal mode of action of ozone is solely an addition with no secondary oxidation. Furthermore, the hydrogen atoms attached to unsaturated centers are left undisturbed.

Two cc quantities of the selected sera were subjected to ozonized oxygen having a concentration of approximately one part ozone to 1000 parts of oxygen. Volume concentrations were ascertained by the iodine liberation technique in which free I is liberated from

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1 Boyd, William C., *J. Exp. Med.*, 1946, **83**, 221.

2 Tyler, A., *J. Immunol.*, 1945, **51**, 157.

TABLE I
Effect of Ozone on Anti-A Isoagglutinin Titer.

Anti-A isoagglutinating serum	Serum dilutions 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Untreated control	+++	+++	+++	+++	+++	+++	+++	++	+	±	—
T-4 min.	+++	++	+	±	—	—	—	—	—	—	—
T-8 "	—	—	—	—	—	—	—	—	—	—	—

+++, ++, +, ± = Varying degrees of agglutination of Group A erythrocytes.
— = No agglutination.
T = treated with ozonized oxygen.

TABLE II
Effect of Ozone on Anti-B Isoagglutinin Titer.

Anti-B isoagglutinin serum	Serum dilutions 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Untreated control	+++	+++	+++	+++	+++	+++	++	++	+	+	—
T-4 min.	+++	+++	+++	++	+	±	—	—	—	—	—
T-8 "	+++	+++	+++	++	+	—	—	—	—	—	—
T-10 "	+++	+++	+++	++	+	—	—	—	—	—	—
T-14 "	—	—	—	—	—	—	—	—	—	—	—

+++, ++, +, ± = Varying degrees of agglutination of Group B erythrocytes.
— = No agglutination.

TABLE III
Effect of Ozone on Anti-A Titer of Ascaris Immune Serum.

Anti-A ascaris immune serum	Serum dilutions 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Untreated control	+++	+++	+++	+++	+++	++	+	+	+	+	—
T-10 min.	+++	+++	+++	+++	++	+	—	—	—	—	—
T-15 "	—	—	—	—	—	—	—	—	—	—	—

+++, ++, +, ± = Varying degrees of agglutination of Group A erythrocytes (human).
— = No agglutination.

KI by O_3 in an acidic solution. Iodine in turn was measured by titration with standard thiosulphate using starch as an indicator. Gas flow was regulated to one liter per 3 minutes of time. Calibration of flow before and after treatment of the specimen was done using a reliable gas meter.

The serum was placed in a 75 cc beaker in order to have ample space for the resulting foam, and the gas delivered by means of a drawn glass tip. The foam was agitated to make available new foam to the active agent.

The manufacture of the ozone from O_2 was accomplished with an electrical ozonizer developed by one of us (Fenning).[3] The output of a modulated 125 megacycle oscillator activated with D.C. pulses with a repetition frequency of 125/sec having a duration of 10 microseconds and a peak power of 2000 Watts was used to activate the ozonizer.

The anti-A and anti-B typing sera used in this work were obtained from group B and A subjects respectively who had been stimulated with group A and B substance by the method of Witebsky *et al.*[4] These sera were of high titer and avidity. Anti-A immune serum prepared by inoculating rabbits with saline extracts from the cuticle of *Ascaris lumbricoides* was also used in these tests. This anti-A immune rabbit serum was of high titer and reacted specifically against human group A erythrocytes but exhibited no titer against group B or O erythrocytes.

The Rh antiserum used was the anti-D (Rh_o) 85% specificity and was absorbed and processed in the Blood Grouping Laboratory in the Department of Bacteriology at the University of Utah. It had a titer of 1:128.

All erythrocyte suspensions used were of 2% strength in terms of blood sediment. They were washed once in physiological saline and resuspended. Physiological saline was used as the diluent of both sera and erythrocytes in all tests except those in which "blocking" serum was used. In these blocking tests bovine albumin, produced by Armour and Company, was used as the diluent for both erythrocytes and serum.

Results and Discussion. Results of these tests are shown in the tables. From the data shown in Table I it is apparent that the group A isoantibody was partially destroyed in 4 minutes treatment with ozone and completely destroyed by 8 minutes of exposure.

A group A anti-B serum of identical titer and avidity similar to the anti-A serum was treated with ozone, and the results of this experiment are shown in Table II.

From the data presented in Table II it is apparent that the group B isoantibody was completely destroyed in 14 minutes and partially destroyed in lesser periods of time.

Anti-A immune serum produced in rabbits by inoculating them with cuticle from *Ascaris lumbricoides* was treated in a similar manner as were anti-A and anti-B isoagglutinin sera. The results of this experiment are shown in Table III.

The evidence in Table III indicates that the anti-A titer of Ascaris immune serum was lost following 15 minutes of exposure and partially lost during lesser periods of exposure.

The effect of ozone on anti-D (Rh_o) serum is shown in Table IV.

With reference to Table IV, it is apparent that progressive partial loss of titer was present in all sera tested. Furthermore, our observations show a complete loss of agglutinating and blocking antibody titer with suitably prolonged exposures in all sera of all types so far tested.

It may also be observed that whereas the "bivalent," "heat labile," "agglutinating," "complete," D (Rh_o) antiserum was destroyed by 4 minutes treatment it took 20 minutes of the same treatment to inactivate the "univalent," "heat stable," "blocking," "incomplete," D (Rh_o) antibody. These results are in agreement with the observations of other authors[5,6] with respect to the action of physical factors such as temperature and pressure on these 2 kinds of antisera.

[3] Demonstrated A.A.A.S. exhibit, Dec., 1946, Boston, Mass.

[4] Witebsky, E., Klendshoj, N. C., and McNeil, C., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 167.

[5] Diamond, Louis K., *J. Lab. and Clin. Med.*, 1945, **24**, 122.

[6] Boyd, William C., *J. Exp. Med.*, 1946, **83**, 401.

TABLE IV.
Effect of Ozone on the Titer of Anti-D (Rh_o) Typing Serum.

Rh_o Agglutinating & blocking antibody serum	2% Erythrocyte suspensions in saline	Serum dilutions in saline									
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Untreated control	O Rh′	—	—	—	—	—	—	—	—	—	—
Rh_o Agglutinating	O Rh″	—	—	—	—	—	—	—	—	—	—
serum	O Rh_o	+++	+++	+++	+++	+++	+++	+++	++	—	—
T-4 min.	O Rh′	—	—	—	—	—	—	—	—	—	—
Rh_o agglutinating	O Rh″	—	—	—	—	—	—	—	—	—	—
serum	O Rh_o	—	—	—	—	—	—	—	—	—	—
	2% Erythrocyte susp. in albumin	Serum dilutions in bovine albumin									
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Control	O Rh′	++	+	+	+	—	—	—	—	—	—
Rh_o "Blocking"	O Rh_2	+++	++	++	++	++	+	+	±	—	—
serum	O Rh_o	+++	+++	+++	+++	++	+	+	±	—	—
T-10 min.	O Rh′	—	—	—	—	—	—	—	—	—	—
Rh_o "Blocking"	O Rh_2	+++	+++	+++	+++	++	+	±	—	—	—
serum	O Rh_o	+++	+++	+++	++	+	±	—	—	—	—
T-20 min.	O Rh′	—	—	—	—	—	—	—	—	—	—
Rh_o "Blocking"	O Rh_2	±	±	—	—	—	—	—	—	—	—
serum	O Rh_o	±	—	—	—	—	—	—	—	—	—

+++, ++, +, ± = Varying degrees of Group O Rh cells.
— = No agglutination.

TABLE V.
Effect of Ozone in Converting Anti-Rh_o Agglutinating Antibody to Blocking Antibody.

Treatment of anti-Rh_o agglutinating serum	Test erythrocytes 2% suspension in saline	Serum dilutions in saline 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Untreated	O Rh′	—	—	—	—	—	—	—	—	—
	O Rh_2	+++	++	+	+	+	—	—	—	—
	O Rh_o	+++	+++	+++	+++	++	+	+	—	—
	O Rh–	—	—	—	—	—	—	—	—	—
Treated 13 min. with ozone	O Rh′	—	—	—	—	—	—	—	—	—
	O Rh_2	—	—	—	—	—	—	—	—	—
	O Rh_o	—	—	—	—	—	—	—	—	—
	O Rh–	—	—	—	—	—	—	—	—	—
1 drop treated serum plus 1 drop untreated	O Rh′	—	—	—	—	—	—	—	—	—
	O Rh_2	+++	+++	++	++	+	+	—	—	—
	O Rh_o	+++	+++	++	++	+	+	±	—	—
	O Rh–	—	—	—	—	—	—	—	—	—

+++, ++, +, ± = Varying degrees of agglutination of Group O Rh cells.
— = No agglutination.

TABLE VI.
Comparative Effects of Air, Oxygen, and Ozonized Oxygen on Anti-Rh″ Agglutinating Serum.

Treatment of anti-Rh″ agglutinating serum	Test erythrocytes 2% in saline	Serum dilutions in saline Undil.	1:2	1:4	1:8	1:16	1:32
Untreated control	Rh′	—	—	—	—	—	—
	Rh″	+++	+++	++	+	—	—
	Rh_o	—	—	—	—	—	—
10 minutes exposure to air	Rh′	—	—	—	—	—	—
	Rh″	+++	++	+	—	—	—
	Rh_o	—	—	—	—	—	—
10 minutes exposure to oxygen	Rh′	—	—	—	—	—	—
	Rh″	+++	+++	+	—	—	—
	Rh_o	—	—	—	—	—	—
10 minutes exposure to ozonized oxygen	Rh′	—	—	—	—	—	—
	Rh″	+	—	—	—	—	—
	Rh_o	—	—	—	—	—	—

+++, ++, +, ± = Varying degrees of agglutination of Rh″ cells.
— = No agglutination.

The question still remained as to whether treatment with ozonized oxygen would convert the "bivalent" anti-Rh "agglutinating" antibody to "univalent" "blocking" antibody. Boyd[1] failed to produce such a change by exposing agglutinating sera to photoflood lights. To study the effect that ozonized oxygen would exert in this direction, anti-Rh_0 agglutinating serum, having an end titer of 1:64 against a 2% saline suspension of group O Rh_0 erythrocytes, was treated and titrated in the same manner as were the serum specimens in our previous tests. The agglutinating antibody in this serum was completely destroyed by a 13-minute treatment with ozonized oxygen. After titration had shown that the Rh_0 antibody had been destroyed, there was added to each dilution of serum and erythrocyte suspension one drop of similarly diluted untreated anti-Rh_0 serum; the tube containing the 1:2 dilution of treated serum received an additional drop of 1:2 untreated serum, the 1:4 dilution received a drop of 1:4 serum and so on. Each tube now contained one drop of treated and diluted serum, one drop of 2% group O Rh_0 erythrocyte suspension, and one drop of untreated serum of the same dilution as the treated serum. The contents of the tubes were mixed by shaking the rack containing the tubes and all were then again incubated in the 37°C water bath. The results of this test are shown in Table V.

It is clear from Table V that, although the agglutinating antibody was destroyed by treatment with ozone, there was nothing produced in it which inhibited the action of the untreated anti-Rh_0 agglutinating serum when, subsequently, one drop of each dilution of the untreated serum was added to each tube and then incubated in the water bath at 37°C for an additional hour. There appears to be no conversion of anti-Rh_0 agglutinating antibody to anti-Rh_0 blocking antibody by treatment with ozone.

In order to evaluate the potential destructive effects of foaming on proteins and related isohemagglutinins, anti-Rh″ serum was subjected to equivalent 10-minute flows of air, O_2, and ozonized O_2. The control agglutinating titer was 1:8. Following the bubbling of air the agglutinating titer was 1:4; for oxygen, 1:4; whereas the agglutinating titer for comparable exposure to ozonized oxygen was 1+ for the undiluted treated specimen. It will be noted in Table VI that some change was evident in the terminal titer. The intensity of agglutination for the lower dilutions showed no change with air and oxygen whereas a profound change was present with ozonized oxygen. Oxidation as effected by ozone in combination with oxygen is more potent than oxygen alone.

With the use of ozonized oxygen considerable macroscopic evidence of denaturation was evident. In most instances with prolonged exposure there developed a gummy, gelatinous precipitate which adhered to the glass rod and sides of the container. With short exposures macroscopic evidence of this precipitate was frequently absent.

Summary: It has been demonstrated that the blood group isoagglutinins, immune anti-A agglutinin, and Rh agglutinating and blocking antibodies are inactivated or destroyed relatively rapidly with 1:1000 dilution of ozone in oxygen.

So far as Rh agglutinating and blocking antibodies are concerned, our results are in essential agreement with the observations of others with respect to the action of physical factors such as temperature and pressure.

There appears to be no conversion of Rh_0 agglutinating antibody to Rh_0 blocking antibody by treatment with ozone.