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The Inhibition of Immune Haemolysis by Salicylaldoxime

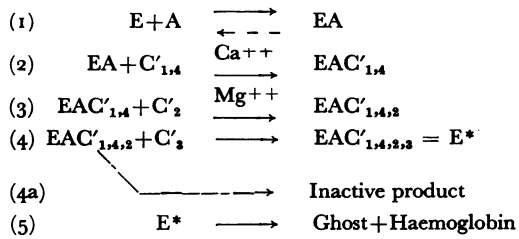
By S. E. Mills and L. Levine

Brandeis University, Waltham, Massachusetts

Summary. It has been shown that haemolysis of sheep erythrocytes by antibody and either guinea pig or human complement is inhibited by salicylaldoxime (*o*-hydroxybenzaldoxime.) Salicylaldoxime differs in its action on immune haemolysis from such previously described inhibitors as ethylenediaminetetraacetic acid and diisopropylfluorophosphate in that it specifically inhibits the reaction between the intermediate complex $EAC'_{1,4,2}$ and C'_3. The inhibition does not appear to be reversible by divalent cations. To be effective the inhibitor must be present during the course of the reaction, since haemolysis proceeds unimpaired if the inhibitor is added to complement and removed by dialysis, or if added to either red cells or $EAC'_{1,4,2}$ and removed by centrifugation. The experimental evidence suggests that salicylaldoxime acts by preventing the effective combination of C'_3 with $EAC'_{1,4,2}$, and not by the destruction of C'_3.

INTRODUCTION

Previous work on the mechanism of sheep erythrocyte lysis by antibody and guinea pig complement (C′) has established the following minimum number of separable steps (Mayer, 1958):



In this schematic representation E represents sheep cells, A antibody, and C'_j various components of complement. The symbol $EAC_{j,k}$, implies merely a state of reactivity of the cell. It is not to be inferred that the components C'_j and C'_k are physically attached to the cell.

Studies on the effect of various inhibitors of immune haemolysis have markedly assisted the clarification of this scheme. For example, analysis of the inhibition caused by ethylenediaminetetraacetic acid (EDTA) led to the recognition and separation of steps (2) and (3), involving Ca^{++} and Mg^{++} ions (Levine, Osler and Mayer, 1953), while studies on the inhibitory action of diisopropylfluorophosphate yielded evidence on the nature of the esterase activity of C'_1 (Levine, 1955; Becker, 1956).

During the course of some investigations on certain possible metal cofactors of immune haemolysis we have come upon another inhibitor, salicylaldoxime (SALDOX). Some of the properties and characteristics of this inhibition form the substance of the following report.

MATERIALS

COMPLEMENT (C′)

Pools of guinea pig and human serum were used as sources of complement. The serums were dispensed in 5 to 10 ml. amounts into pyrex screw-cap tubes, and stored at −20° in an electric deep freeze.

RED CELLS

Sheep erythrocytes were purchased from Carworth farms as suspensions in Alsever's solution, and stored at 2° to 5° in a refrigerator. Before use the cells were washed, standardized, and sensitized with antibody as described by Osler, Strauss and Mayer (1952).

ANTIBODY

The haemolysins used in these experiments were of two kinds. The first, D79, was prepared by the injection into rabbits of washed, boiled, sheep erythrocyte stromata. The other, CL-1128, purchased from Cappell Laboratories, was obtained from rabbits by the injection of whole red cells.

BUFFERS

Stock veronal buffer, *p*H 7·4, containing 5×10^{-4} MMg^{++} and $1{\cdot}5\times10^{-4}$, MCa^{++} was prepared as described by Mayer, Croft and Gray (1948). Serum albumin buffer (SA-buffer) was made from veronal by the addition of 0·1 per cent (w/v) bovine serum albumin.

SALICYLALDOXIME (SALDOX)

Salicylaldoxime was purchased from Merck Chemical Company. Fresh solutions of 0·01 M SALDOX in buffer were prepared immediately before each experiment. When higher concentrations were required, 1·0 M stock solutions were prepared in 95 per cent ethanol and diluted in buffer. In the latter event, appropriate ethanol diluent controls were included in the experiment.

In the experiments to be described the extent of lysis of a suspension of sheep erythrocytes was determined in a Beckman spectrophotometer, model DU, by measurement of the optical density (O.D.) of the released oxyhaemoglobin in the supernatant at a wavelength of 541 mμ against distilled water as a reference standard.

EXPERIMENTAL PROCEDURES AND RESULTS

EXPERIMENT I: THE EFFECT OF SALDOX ON IMMUNE HAEMOLYSIS

The original observations on the inhibition of immune haemolysis by SALDOX were obtained from the following experiment. A standard suspension of sheep cells was sensitized with rabbit hemolysin CL-1128, diluted 1 to 2000 in buffer. This dilution of haemolysin was at least four times in excess of the amount necessary to give maximum lysis under the conditions of the experiment. To a series of 40 ml. test tubes in an ice-bath

were added 5×10^8 sensitized red cells, increasing concentrations of SALDOX, and approximately one $C'H_{50}$ unit of either guinea pig or human complement. One $C'H_{50}$ unit is that amount of complement sufficient to cause lysis of 50 per cent of the cells. The final volume in all tubes was 7·5 ml. Controls were included to determine the extent of spontaneous lysis of the cells, and the effect of SALDOX alone on the cells. The reaction mixtures were incubated at 37° for one hour with intermittent shaking, centrifuged for 10 minutes at $1000\times g$, and the extent of lysis determined in the spectrophotometer. The percent inhibition at each concentration level of SALDOX was recorded as

$$100\left(1-\frac{\text{O.D. C'+SALDOX}}{\text{O.D. C'}}\right)$$

The results obtained for both human and guinea pig complement are presented in Fig. 1. As can be seen from the graph, inhibition was first detected at about 1×10^{-4} M SALDOX, increased rapidly to 45–55 per cent inhibition at $5{\cdot}0\times10^{-4}$ M SALDOX, then increased more slowly at still higher levels of the inhibitor.

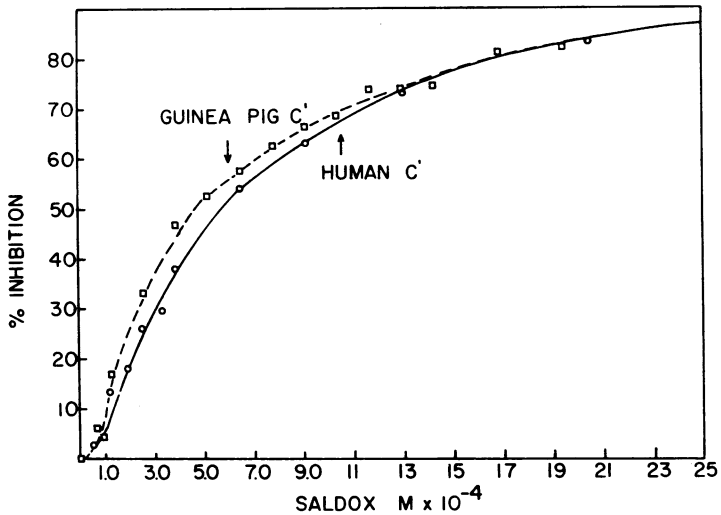


FIG. 1. The effect of salicylaldoxime on immune haemolysis. The test system contained 5×10^8 EA and one $C'H_{50}$ unit in a final volume of 7·5 ml. Percent inhibition was recorded as $100\left(1-\frac{\text{O.D. C'+SALDOX}}{\text{O.D.C'}}\right)$

EXPERIMENT II: THE EFFECT OF VARIOUS METAL IONS ON INHIBITION OF IMMUNE HAEMOLYSIS BY SALDOX

Salicylaldoxime is a known chelating agent of various divalent metal ions, with a strong affinity for Cu^{++}, Ni^{++} and Fe^{++} (Martell and Calvin, 1952). Since it has been clearly demonstrated that Ca^{++} and Mg^{++} are necessary for immune haemolysis with guinea pig C' (Levine *et al.*, 1953), experiments were designed to determine whether SALDOX acted by binding Ca^{++} and Mg^{++}. It was found that the addition of Ca^{++} and Mg^{++} did not reverse the inhibition caused by SALDOX. In addition, as will be shown in Experiment V, the intermediate product $EAC'_{1,4,2}$ could be recovered from the reaction between sensitized cells, complement, and SALDOX. Since the formation of $EAC'_{1,4,2}$ is

dependent upon the presence of Ca^{++} and Mg^{++} (Levine, Mayer and Rapp, 1954), it was evident that chelation of these two metal ions was not involved in the inhibition.

The possibility was then explored that other metal ions could reverse the effect of SALDOX, and perhaps participate in immune haemolysis. To a series of test tubes were added 5×10^8 EA, sensitized as in Experiment I, $6{\cdot}7\times10^{-4}$ M SALDOX, approximately 1 $C'H_{50}$ unit (guinea pig), and varying concentrations of the metal ions to a final volume of 7·5 ml. The metal salts were dissolved in either veronal buffer or saline, and the amounts tested ranged from a final concentration of $1{\cdot}3\times10^{-6}$ M to $6{\cdot}7\times10^{-3}$ M, ten times the concentration of the SALDOX employed. Controls were included to measure the effect of each metal on the sensitized cells alone and on lysis by C'. The following salts: $MnSO_4$; $ZnSO_4$; $Co(NO_3),6H_2O$; $Na_2B_4O_7,10H_2O$ and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, were ineffective in reversing SALDOX inhibition. However, the addition of Cu^{++}, Ni^{++}, or Fe^{++} produced somewhat surprising results in that, at sufficiently high concentrations of any of the metal ions, the cells were lysed. For example, 2×10^{-5}M Cu^{++} and $6{\cdot}7\times10^{-4}$ M SALDOX lysed 5×10^8 sensitized cells with or without the addition of complement; and, as further experiments revealed, independently of the presence of antibody on the cells. The lytic action of SALDOX and Cu^{++} was rapid at 37°, occurring within 3 to 5 minutes, but did take place at 0° after more extended periods of incubation. Addition of Cu^{++} first, followed by SALDOX also was effective. However, mixing the metal with the chelator before addition resulted in little or no lysis. Essentially similar results were obtained with Ni^{++} and Fe^{++}, although different metal-to-chelator ratios was found necessary to cause lysis of a given number of cells. The addition of EDTA to the erythrocytes, under identical experimental conditions, followed by Cu^{++}, Ni^{++}, or Fe^{++}, resulted in no demonstrable haemolysis.

The complications introduced by the non-immune lysis of red cells by SALDOX and either Cu^{++}, Ni^{++}, or Fe^{++} rendered it difficult to assess the possible role, if any, of these ions in the haemolytic inhibition caused by SALDOX. Indirect evidence was obtained, however, that made it appear unlikely that these metal ions had a significant function. As will be presented in greater detail in Experiment VII, *m*-hydroxybenzaldehyde, a compound structurally related to SALDOX but without metal chelating properties, is also a potent inhibitor of immune haemolysis. Both *m*-hydroxybenzaldehyde and SALDOX appear to inhibit by a common mechanism, suggesting that there is no absolute requirement for metal chelation in the inhibition.

EXPERIMENT III: TREATMENT OF CELLS AND COMPLEMENT WITH SALDOX

The lytic effect produced by the successive addition of SALDOX and Cu^{++}, together with the fact that no lysis occurred if the metal chelate was added to the cells, indicated that SALDOX could combine with the red cells, and, presumably, in such a manner that the chelating portion of the molecule remained free to react with the added metal.

An attempt was made to measure the amount of SALDOX in combination with the cells by taking advantage of the fact that solutions of the inhibitor in veronal buffer at *p*H 7·5 were found to have an absorption peak at 305 mμ, and obey Beer's Law up to a concentration of $3{\cdot}3\times10^{-3}$ M. To a series of tubes containing 10^9 cells suspended in 7·5 ml. of veronal buffer were added concentrations of SALDOX ranging from $1{\cdot}0\times10^{-5}$ M to $3{\cdot}3\times10^{-3}$ M. A control series contained identical concentrations of SALDOX in veronal. All tubes were incubated at 37° for 30 minutes, centrifuged at $1000\times g$ for 5

minutes, and the reaction mixtures read at 305 mμ in the spectrophotometer. Analysis revealed no significant difference in SALDOX concentration between the control series and the supernatant fluids from the tubes containing the cells. The standard error of the measurement was approximately $1{\cdot}3\times10^{-2}$ μM of SALDOX per ml. If the standard error is taken as a measure of the maximum amount of SALDOX combining with the cells, the addition of up to $1{\cdot}5\times10^{10}$ molecules of SALDOX per red cell resulted in the combination of a maximum of 6×10^{7} molecules of SALDOX per red cell. It was evident that the bulk of the inhibitor remained in the medium, although by inference a small amount, sufficient for metal-chelator haemolysis, must have combined with the cells.

Since a negligible portion of the inhibitor was bound to the red cells, the remainder could react with complement directly and destroy its activity. To test this possibility three reaction mixtures containing 1·5 $C'H_{50}$ units of guinea pig C′ per ml. and 0, 0·005, and 0·009 M SALDOX respectively were set up and incubated at 37°. Samples were withdrawn at 0, 15, 30, and 45 minutes, and dialysed overnight at 4° against saline containing optional concentrations of Ca^{++} and Mg^{++}. After dialysis the reaction mixtures were examined for complement activity with 5×10^{8} sensitized cells. The concentrations of C′ and SALDOX employed were such that a minimum of 75 per cent of the complement activity would have been destroyed if, indeed, SALDOX did inhibit by the direct destruction of complement.

The results of this experiment are presented in Table 1.

TABLE 1

THE EFFECT ON GUINEA PIG C′ OF PREINCUBATION WITH SALDOX FOLLOWED BY DIALYSIS. C′ (1·5 $C'H_{50}$ UNITS PER ML.) WAS INCUBATED AT 37° WITH SALDOX, SAMPLED AT VARIOUS TIMES, DIALYSED OVERNIGHT AGAINST SALINE PLUS CA^{++} AND MG^{++}, AND TESTED FOR ACTIVITY WITH 5×10^{8} EA IN A FINAL VOLUME OF 7·5 ML.

Time of Sampling (min.)	*Series 1* C′ *O.D.*	*Series 2* $C'+0{\cdot}5\times10^{-2}M$ *SALDOX* *O.D.*	*Series 3* $C'+0{\cdot}9\times10^{-2}M$ *SALDOX* *O.D.*
0	0·465	0·500	0·450
15	0·495	0·470	0·445
30	0·486	—	0·387
45	0·418	0·418	0·365

O.D. = Optical Density.

The loss of activity of the control, if computed on the basis of a zero time reading of 500, was 16 per cent at the end of 45 minutes. The figure of 500 was taken as the zero time O.D., since it seemed more reasonable that a sampling error occurred to give the recorded value of 0·465 than that the activity of C′ increased during the 15 minute incubation period at 37°. There was a 16 per cent reduction in activity for series 2, initially containing $0{\cdot}5\times10^{-2}$ M SALDOX, and 19 per cent loss in series 3, with $0{\cdot}9\times10^{-2}$ M SALDOX. It was apparent that SALDOX caused no significant loss of C′ activity when incubated with guinea pig serum alone.

A similar analysis was made of the effect of SALDOX on sensitized red cells. A flask containing 5×10^{8} sensitized cells per ml. was incubated with 5×10^{-3} M SALDOX at 37°. At time intervals up to one hour 1·0 ml. samples were withdrawn. The samples were

centrifuged, the supernatants discarded, and the cells tested with one guinea pig $C'H_{50}$. It was found that the cells lysed as readily as a control series which had not been in contact with SALDOX.

These experiments showed that treatment of either sensitized cells or guinea pig serum with the inhibitor, followed by its removal, had no permanent effect on the capacity of these reagents to react in immune haemolysis.

EXPERIMENT IV: THE INHIBITION BY SALDOX OF COMPLEMENT ACTIVITY DURING THE COURSE OF HAEMOLYSIS

Attention was then turned to the possibility of isolating the precise reaction in the haemolytic sequence that was sensitive to the action of the inhibitor. To obtain information on this point the experimental procedure developed by Mayer and Levine (1954a), was employed to follow the time-course of haemolysis of sensitized cells by C^1. Briefly, in this procedure a number of suspensions of sensitized cells are placed on a shaker in a 37° water bath. Appropriate dilutions of C′ and, as required, other reactants are then added. The addition of C′ to one of the cell suspensions is chosen as zero time, and serves as a reference for all further additions and sampling. When samples are withdrawn they are immediately pipetted into twice the sample volume of ice-cold isotonic 'stop' solution containing 0·01 M EDTA. Dilution at 0° stops the formation of $EAC'_{1,4,2,3}$ (E*) from $EAC'_{1,4,2}$ and C'_3, while EDTA arrests additional formation of $EAC'_{1,4,2}$. Upon completion of the experiment the tubes are centrifuged in the cold and reincubated at 37° for one hour without disturbing the pellet. The tubes are then chilled, the pellet resuspended, and the samples centrifuged and analysed for oxyhaemoglobin.

It has been shown by Mayer and Levine (1954b) that the reaction E*→ ghost+ haemoglobin is relatively slow. When a sample is taken it contains cells that have already lysed, together with cells in the state E*. The procedure outlined above permits the expression of E* and, thereby, the determination of all the cells that had reacted to form E*, not merely those already lysed. In these experiments both human and guinea pig C′ and haemolysins CL-1128 and D79 were employed. Since the results were indistinguishable for all combinations of C′ and haemolysin, the data given below refer only to guinea pig C′ and D79. In addition, two different relative proportions of haemolysin and C′ were employed. For example, in the system designated excess antibody-limited C′, guinea pig C′ was used as a 1 to 100 dilution in buffer, and the cells were sensitized with a 1 to 250 dilution of D79, an amount of antiserum at least 20 times in excess of that necessary to give maximum haemolysis. In the second system, designated excess C′–limited antibody, C′ was diluted 1 to 15, and the haemolysin 1 to 8000. The number of cells, as well as all other reactants, was the same in both systems. In a typical experiment 5×10^9 cells sensitized in antibody excess were suspended in a number of flasks. C′ was added at a level of 7·5 $C'H_{50}$ units, and SALDOX at a concentration of 4×10^{-3} M. The final volume was 25 ml. The results of the kinetic experiments are presented in Fig. 2. The data reveal that the addition of SALDOX at any time during the course of active haemolysis in the excess antibody–limited C′ system caused marked inhibition. The same result was obtained in the excess C′–limited antibody system. This is in contradistinction, for example, to the effect produced by EDTA (Mayer, 1958). The addition of EDTA at any time to an excess C′–limited antibody system promptly stops haemolysis; but to inhibit the excess antibody–limited C′ system, EDTA is effective only if added within one

or two minutes after the addition of C′. The effect of EDTA has been interpreted to mean that in excess antibody-limited C′, C'_1, C'_4, and C'_2 add rapidly to EA, and, since the lysis of $EAC'_{1,4,2}$ by C'_3 is not cation dependent, the system rapidly becomes insensitive to inhibition by EDTA. In excess C′-limited antibody, however, new $EAC'_{1,4,2}$ sites (mediated by Ca^{++} and Mg^{++}) are presumably forming throughout the entire course of the reaction, thereby rendering the system vulnerable at all times to EDTA.

Based on this interpretation, the inhibition pattern of SALDOX leads to the conclusion that inhibition occurred after the formation of $EAC'_{1,4,2}$. It may be noted that the addition of SALDOX to an excess antibody-limited C′ system in which 85 per cent lysis had occurred was still effective in preventing maximum lysis of the cells.

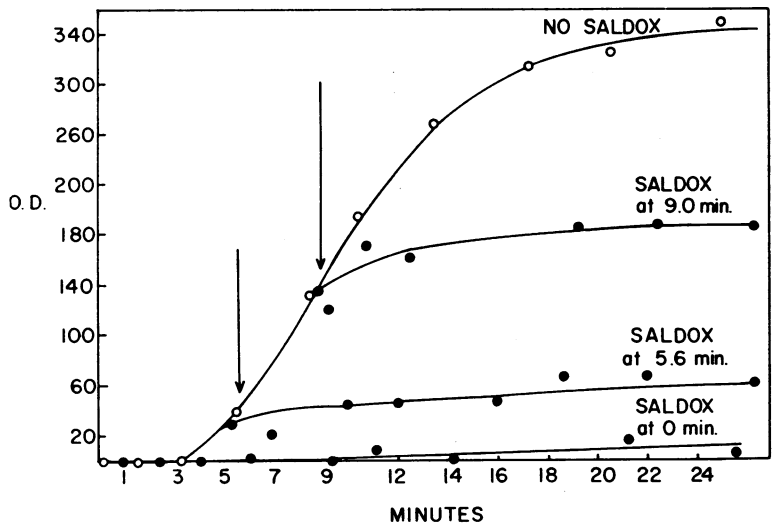


FIG. 2. Kinetics of haemolysis with excess antibody and limited complement. The reaction mixture contained 5×10^8 EA, sensitized with a 1 to 250 dilution of haemolysin D79, 7·5 guinea pig $C'H^{50}$ units, and SALDOX, added at the indicated times, at a final concentration of 4×10^{-3} M. The final volume was 25 ml.

Examination of Fig. 2 reveals an additional effect. While the addition of SALDOX did result in a dramatic decrease in the velocity of haemolysis, haemolysis was not entirely eliminated. A small but persistent increase in lysis continued with time in both systems. A possible explanation was that SALDOX was slowly inactivated by one of the components of the system. However, experiments revealed that SALDOX did not measurably deteriorate in veronal buffer for up to 45 minutes at 37°. In addition, when the inhibitor was incubated with guinea pig serum at 37° and samples withdrawn and compared with a parallel control serum for activity, no significant change in inhibition occurred during the course of the 45 minutes' incubation period.

EXPERIMENT V: THE FORMATION OF $EAC'_{1,4,2}$ BY EA AND C′ IN THE PRESENCE OF SALDOX

The inference drawn from the preceding experiment, that inhibition occurred after the formation of $EAC'_{1,4,2}$, suggested that an intermediate product could be isolated from the reaction between EA, C′, and SALDOX. To test the possibility that this reaction

product was E*, the following experiment was performed. To an Erlenmeyer flask were added 10 ml. of buffer followed by 10 ml. of EA (5×10^8 per ml.) sensitized in antibody excess with CL-1128. The reaction mixture was placed on a shaker in a 37° water bath, and 5·0 ml. of guinea pig C′ (7·5 $C'H_{50}$) added. At various time intervals during the course of the reaction samples were taken and pipetted into ice-cold 'stop' solution. The samples were immediately centrifuged in the cold, and the supernatants poured off and analysed for oxyhaemoglobin. The sedimented cells from the first, third, fifth, and all subsequent odd-numbered samples were resuspended in veronal containing 5×10^{-3} M SALDOX (a concentration sufficient for at least 90 per cent inhibition) and 0·01 M EDTA; the cells from the even-numbered samples were resuspended in veronal plus 0·01 M EDTA without SALDOX. The resuspended cells were incubated for 1 hour at 37°

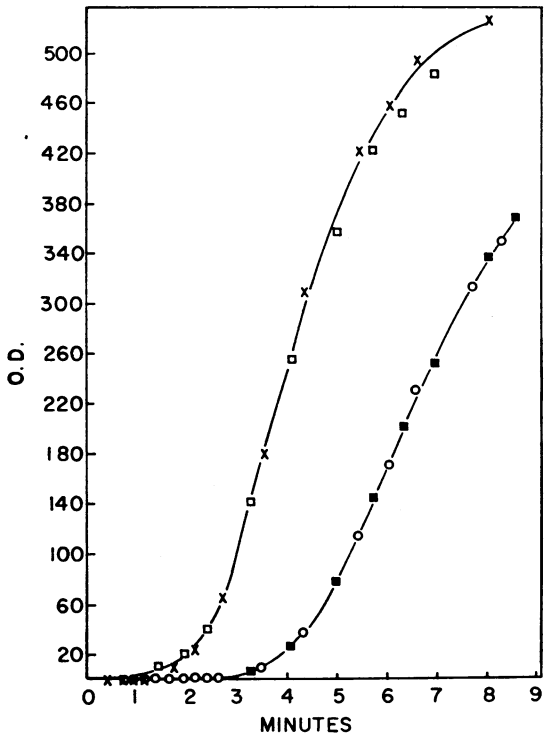


FIG. 3. The effect of SALDOX on $EAC'_{1,4,2,3}$ (E*). During the course of haemolysis of a single reaction mixture, composed of EA and guinea pig C′, samples were withdrawn, sedimented, the supernatant fluid removed, and the cells in the state E* permitted to lyse at 37° in 0·01 M EDTA-saline or 0·01 M EDTA-saline plus 0·005 M SALDOX.

O — the O.D. of the supernatant fluid of each sample of E* resuspended in EDTA-saline.

■ — the O.D. of the supernatant fluid of each sample of E* resuspended in EDTA-saline plus SALDOX.

X — the sum of the O.D. of the supernatant and the corresponding sediment after the lysis of E* in EDTA-saline.

□ — the sum of the O.D. of the supernatant and the corresponding sediment after the lysis of E* in EDTA-saline plus SALDOX.

to permit the expression of E*, then analysed for haemolysis. The optical density of each supernatant was added to that obtained from the corresponding cell sediment (E*), and the sum plotted against the time of sampling as shown in Fig. 3. A plot of the supernatant optical density alone is included in the figure. From the results it can be seen that SALDOX does not inhibit the lysis of E*, since a smooth curve represents both odd- and even-numbered samples. If inhibition at the E* level did occur, a distinct curve would have been obtained for the odd-numbered samples. This curve would lie below the curve actually found and coincide with the one obtained for the supernatants alone, since sufficient SALDOX was added to the resuspending medium to prevent the lysis of all but a negligible fraction of the cells.

Since inhibition of E* lysis was ruled out, the next experiment was designed to deter-

TABLE 2

FLOW DIAGRAM FOR THE DETERMINATION OF $EAC'_{1,4,2}$ FROM THE REACTION BETWEEN EA, C', AND SALDOX

EA+SALDOX+C'
Shaken at 37° and sampled twice at each time period

Sample 1 (1·5 ml.) → Into 3·0 ml. EDTA-Stop at 0°

Sample 2 (1·5 ml.) → Into 3·0 ml. EDTA-Stop at 0° C; immediately centrifuged at 0°

→ Supernatant

→ Pellet: resuspended in 1·0 ml. C' (diluted 1 to 60) + 3·5 ml. EDTA-Stop

mine whether $EAC'_{1,4,2}$ could be detected as a product of the reaction between C', EA, and SALDOX. In Table 2 a flow diagram is given for the analysis of the reaction between EA, C', and SALDOX. A duplicate reaction without the inhibitor was analysed simultaneously, and the usual controls were included to measure the spontaneous lysis of the cells and the effect on haemolysis of centrifugation and resuspension.

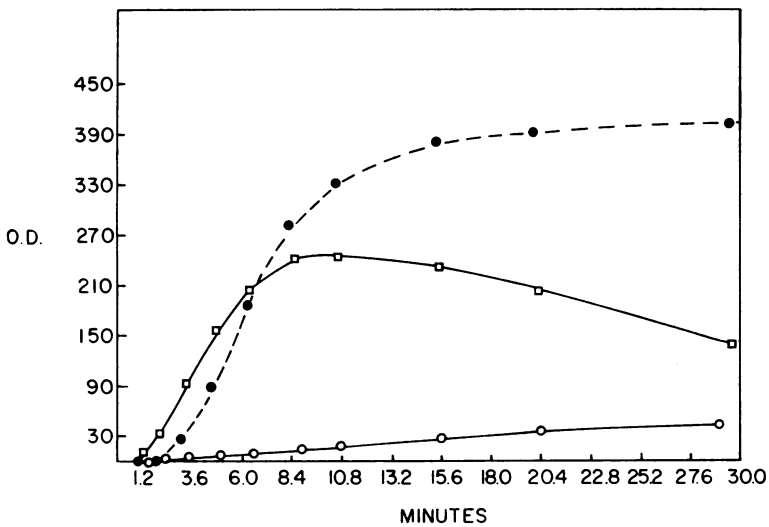


FIG. 4. Kinetics of the formation of $EAC'_{1,4,2}$ in the presence of SALDOX. The reaction mixture contained 10^{10} EA, 15 guinea pig $C'H_{50}$ units, and SALDOX at 4×10^{-3} M, all in a final volume of 50 ml.
●—● Haemolysis of a control reaction without SALDOX.
O—O Haemolysis in the presence of SALDOX.
□—□ $EAC'_{1,4,2}$ accumulation in the presence of SALDOX as measured by the lysis caused by C'_2 after the removal of SALDOX.
O.D. Optical density.

The proportions of the reactants used were as follows: (1) EA-20 ml. of cells (5×10^8 per ml.) sensitized with CL-1128 in antibody excess; (2) SALDOX—to a final concentration of 4×10^{-3} M; (3) guinea pig C'—15 $C'H_{50}$ units; (4) SA-buffer—to 50 ml.

Sample 1 was analysed by the usual kinetic procedure which included centrifugation, incubation at 37° for one hour without disturbing the pellet, and analysis for lysis. The results, plotted as curve A in Fig. 4, give the total amount of lysis in the reaction mixture, and, when compared to the control, the extent of inhibition. The pellet of sample 2, containing the unlysed cells at the time of sampling, were resuspended in approximately 2 $C'H_{50}$ units of guinea pig C′ in 0·01 M EDTA. Complement in 0·01 M EDTA serves as a convenient source of C'_3 and would cause lysis of only those cells in the pellet in the state $EAC'_{1,4,2}$. Curve B of Fig. 4 represents the formation with time of $EAC'_{1,4,2}$ (Corrected for E* in the pellet by subtracting the supernatant lysis in sample 2 from the total lysis in sample 1). It can be seen that the number of $EAC'_{1,4,2}$ reached a peak at about 9 minutes, then declined. This can be attributed to the sum of two competing processes—the formation of $EAC'_{1,4,2}$, and its simultaneous decay to an inactive form (Mayer, 1958). Curve C represents the reference kinetic analysis, done at the same time, with identical concentrations of EA and C′ but without SALDOX.

EXPERIMENT VI: THE DECAY OF $EAC'_{1,4,2}$ IN THE PRESENCE OF SALDOX

The demonstration that $EAC'_{1,4,2}$ did accumulate in the presence of SALDOX, and that the lysis of E* was not inhibited, led to an examination of the reaction between $EAC'_{1,4,2}$, C'_3 and the inhibitor. $EAC'_{1,4,2}$ was prepared by treating 80 ml. of EA (5×10^8 cells per ml. sensitized in excess antibody with D79) with 5·0 ml. of undiluted C′ in the presence of 1×10^{-3} M Ca^{++} and 3×10^{-3} M Mg^{++} at 0° for 30 minutes (Levine *et al.*, 1954). The cells were then washed with ice-cold 0·02 M EDTA-saline (*p*H 7·4), stored at 0° for four hours to permit the lysis of the small amount of E* formed, and washed again with ice-cold EDTA-saline. The source of C'_3 was guinea pig serum diluted in 0·02 M EDTA-saline at *p*H 7·4.

It was found, as expected, that the reaction between $EAC'_{1,4,2}$ and C'_3 was directly inhibited by SALDOX.

The effect of SALDOX on C'_3 alone was determined by incubating C'_3 with $5{\cdot}3\times10^{-3}$ M SALDOX (sufficient to give greater than 75 per cent inhibition) for 40 minutes at 37°. The reaction mixture, as well as an untreated C'_3 sample, was dialysed overnight against two changes of 40 times its volume of EDTA-saline. When examined with a fresh lot of $EAC'_{1,4,2}$, the C'_3 activity of the two samples was identical, indicating that SALDOX did not destroy C'_3 activity in guinea pig serum.

Information on the interaction between the inhibitor and $EAC'_{1,4,2}$ was obtained by a study of the effect of SALDOX on the rate of decay of $EAC'_{1,4,2}$. As has been shown by Mayer, Levine, Rapp and Marucci (1954), $EAC'_{1,4,2}$, upon standing, loses its capacity to react with C'_3. To determine whether SALDOX inhibition could be attributed to an increase in the rate of decay of $EAC'_{1,4,2}$ reactivity, the following analysis was performed. Three sets of nine tubes per set were placed in a water bath at 0°, and charged with 5×10^8 $EAC'_{1,4,2}$. Each set received one of the following concentrations of SALDOX: 0, $13{\cdot}4\times10^{-4}$, and $53{\cdot}6\times10^{-4}$ M. Sufficient 0·02 M EDTA-saline was added to give a final volume of 6·5 ml. The tubes were placed in a 37° water bath, and zero time chosen when the contents reached 37°. One ml. of C'_3 (as C′ diluted 1 to 50 in 0·02 M EDTA-saline) was added to the tubes of each set at various time intervals up to 30 minutes. From the time of addition of C_3, each tube was incubated for an additional hour, then centrifuged and the supernatant fluids analysed for oxyhaemoglobin. The results of the

experiment are given in Fig. 5 in which the log of the fraction of cells lysable, at this concentration of C'_3, is plotted against the time of C'_3 addition. From the data it appeared that the addition of SALDOX did result in an increase in the rate of decay of $EAC'_{1,4,2}$ as measured by the number of cells that would lyse when exposed to C'_3 after a given period of incubation.

At least two possibilities could account for this result. One was that SALDOX destroyed the reactive sites on the cell that are symbolized by $EAC'_{1,4,2}$. To examine this possibility

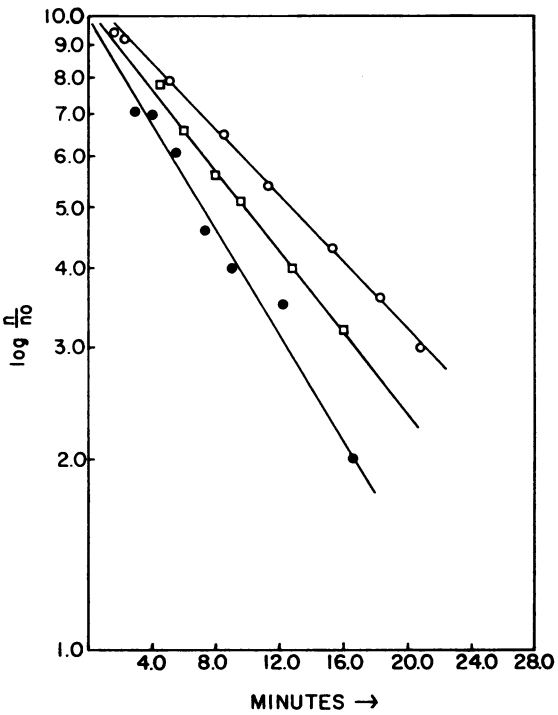


FIG. 5. The decay of $EAC'_{1,4,2}$.
At various time intervals 1·0 ml. of C'_3 (as C′ diluted 1 to 50 in 0·02 M EDTA-saline) was added to 5×10^8 $EAC'_{1,4,2}$ in 6·5 of 0·02 M EDTA-saline. Log n/n_0 represents the log of the fraction of the cells lysable by this concentration of C'_3 at a given time.

O—O Decay with C'_3 at 1 to 50.

□—□ Decay with C'_3 at 1 to 50 plus $13{\cdot}4 \times 10^{-4}$ M SALDOX.

●—● Decay with C'_3 at 1 to 50 plus $53{\cdot}6 \times 10^{-4}$ M SALDOX.

an experiment, identical to the one described above, was performed with two series of tubes. One series contained no inhibitor, while the other contained sufficient SALDOX to inhibit lysis of 50 per cent of the cells. Before the addition of C'_3, however, the cells in both series were rapidly centrifuged, the supernatants discarded, and the packed cells resuspended in 0·02 M EDTA-saline. Under these conditions the rate of decay of $EAC'_{1,4,2}$ proved to be the same. Thus, SALDOX did not increase the rate of destruction of $EAC'_{1,4,2}$.

Another possible explanation for the apparent enhancement by SALDOX of $EAC'_{1,4,2}$ decay involved the method used to measure the decay. It must be emphasized that the method described above measures the rate of decay of the number of *cells* in the $EAC'_{1,4,2}$ state that can react at any time with C'_3. However, the reaction with C'_3 requires time. The addition of C'_3 does not arrest the decay reaction. Decay still proceeds and is in competition with the productive reaction between $EAC'_{1,4,2}$ and C'_3 to form E*. It was possible, therefore, that the rate of decay, as measured above, could be a function of the

C'_3 concentration. If this were true, then the results obtained with SALDOX could be explained by the assumption that SALDOX acted to decrease the effective concentration of C'_3.

To test this possibility, five series of reaction mixtures were set up with 5×10^8 $EAC'_{1,4,2}$. The rate of decay in three of the series was analysed with C'_3, diluted 1 to 40, 1 to 65, and 1 to 90 respectively, but with no inhibitor. A fourth series contained SALDOX at $6{\cdot}7 \times 10^{-4}$ M and the fifth, SALDOX at 20×10^{-4} M, and were both analysed with C'_3 at 1 to 40. The concentrations of the inhibitor were chosen so that at zero time, before appreciable decay had occurred, C'_3 at 1 to 40 plus SALDOX at $6{\cdot}7 \times 10^{-4}$ M caused approximately the same extent of lysis as did C'_3 diluted 1 to 65. Similarly, the higher concentration of SALDOX with 1 to 40 C'_3 gave approximately the same lysis at zero time as did the 1 to 90 dilutions of C'_3.

TABLE 3

MEASUREMENT OF THE RATE OF DECAY OF $EAC'_{1,4,2}$ WITH VARIOUS CONCENTRATIONS OF C'_3 AND SALDOX

Reagents	*Optical density at zero time*	*Half-life of $EAC'_{1,4,2}$ (minutes)*
(1) C'_3 (1 to 40)	0·551	22·0
(2) C'_3 (1 to 65)	0·334	16·4
(3) C'_3 (1 to 40) plus $6{\cdot}7 \times 10^{-4}$ M SALDOX	0·356	15·0
(4) C'_3 (1 to 90)	0·204	12·0
(5) C'_3 (1 to 40) plus 20×10^{-4} M SALDOX	0·215	12·4

The results of the experiment are recorded in Table 3 in terms of the half-life of the $EAC'_{1,4,2}$ preparation. It was apparent that a decrease in the C'_3 concentration resulted in an increase of the rate of decay of the cells as measured by the method described above. At a 1 to 40 dilution of C'_3 the half-life of the cells was 22 minutes, whereas at a 1 to 90 dilution of C'_3 the half-life of the cells was 12 minutes. In addition, C'_3 at 1 to 40 yielded essentially the same rate of decay in the presence of $6{\cdot}7 \times 10^{-4}$ M SALDOX as did C'_3 alone at 1 to 65. Similarly, C'_3 at 1 to 40 plus 20×10^{-4} M SALDOX gave the same rate of decay as C'_3 alone at 1 to 90. From these results, and the fact that SALDOX does not destroy either $EAC'_{1,4,2}$ or C'_3, it can be inferred that the inhibitor acts to diminish the concentration of C'_3 by, in some manner, preventing the effective lytic combination between $EAC'_{1,4,2}$ and C'_3.

EXPERIMENT VII: INHIBITION OF HAEMOLYSIS BY COMPOUNDS STRUCTURALLY SIMILAR TO SALDOX

Several compounds, structurally related to SALDOX, were examined as potential inhibitors of immune haemolysis. The compounds were dissolved in either veronal or saline or, if insoluble, were assayed as suspensions. The assay system was that described in Experiment I. A list of some of these compounds and their activity is shown in Table 4. With 50 per cent inhibition by 5×10^{-4} M SALDOX as a reference point, it can be seen

that *o*-hydroxybenzaldehyde (salicyaldehyde) was about twice as inhibitory as SALDOX, *m*-hydroxybenzaldehyde about one-half as effective, while the activity of *p*-hydroxybenzaldehyde was sharply diminished.

The effect of *m*-hydroxybenzaldehyde was of particular interest, since this compound is not a metal chelator. Further studies with *m*-hydroxybenzaldehyde revealed that, in common with SALDOX, it inhibits the reaction between $EAC'_{1,4,2}$ and C'_3, and permits the isolation of $EAC'_{1,4,2}$ when added to a reaction mixture containing sensitized cells and guinea pig complement.

The activities of phenol, benzaldehyde, and benzaldoxime were of the same order of magnitude, all six to seven times less potent than SALDOX. When the aromatic ring of phenol was replaced by an aliphatic ring, as in cyclohexanol, or when phenol was substituted in the para position, as with p-nitrophenol, activity was sharply reduced. No

TABLE 4

INHIBITION OF IMMUNE HAEMOLYSIS BY COMPOUNDS STRUCTURALLY RELATED TO SALDOX. THE TEST SYSTEM CONTAINED 5×10^8 EA AND ONE GUINEA $C'H_{50}$ UNIT IN A FINAL VOLUME OF 7·5 ML.

Compound	*Inhibition (per cent)*	*Concentration ($M \times 10^{-4}$)*
Salicylaldoxime	50	5·0
o-Hydroxybenzaldehyde	50	2·5
m-Hydroxybenzaldehyde	50	10
p-Hydroxybenzaldehyde	30	33
Benzaldoxime	50	40
Benzaldehyde	50	40
Benzyl alcohol	15	47
Phenol	50	33
p-Nitrophenol	<10	33
Cyclohexanol	<10	47
Benzoic acid	<10	33
Salicylic acid	<10	33
Methyl salicylate	50	33
Salicylamide	50	33

inhibition was observed with the negatively charged benzoic or salicylic acids, although esterification of the carboxyl group in salicylic acid, as in methyl salicylate or salicylamide, restored activity to the level of phenol.

DISCUSSION

The resolution and ordering of the individual reactions in immune haemolysis have been greatly facilitated by analysis of the inhibition exerted on this system by EDTA. Through careful studies with EDTA, the role of Ca^{++} and Mg^{++} ions as co-factors of guinea pig complement was clarified, and this in turn led to the development of methods for the isolation and characterization of various intermediate reaction products.

Up to the present, ions other than Ca^{++} and Mg^{++} have not been implicated in immune haemolysis. The possibility that additional metal ions are involved, particularly as metal-protein or other metal-macromolecular complexes, has however not been excluded. From the report of Vallee (1955), that the Zn^{++}-containing enzyme, yeast alcohol dehydro-

genase, is inhibited by 1,10-phenanthroline but not by EDTA, and the observations of Rothschild, Cori and Barron (1954) that rat liver choline oxidase, while insensitive to EDTA, is inhibited by salicylaldoxime and other Cu^{++} chelators, it is apparent that a complexed metal ion may be invulnerable to attack by EDTA—the most widely studied inhibitor of complement activity—in spite of the strong affinity of the chelator for the free ion.

These considerations led to a search for metal chelators other than EDTA which would inhibit immune haemolysis at reasonably low concentrations and which would not be reversed by Ca^{++} and Mg^{++}. With the demonstration that salicylaldoxime fulfilled these requirements an attempt was made to determine the reaction in haemolysis sensitive to the action of salicylaldoxime, and second, to define the mechanism responsible for the inhibition.

The experimental evidence presented above suggests that the haemolytic reaction inhibited is that between $EAC'_{1,4,2}$ and C'_3. This reaction, however, is not as simple as is represented in the schematic outline in the introduction. Rapp (1958) and Amiraian, Plescia, Cavallo and Heidelberger (1958) have shown that C'_3 consists of at least two components, and there may be at least two reactions between $EAC'_{1,4,2}$ and C'_3. Whether salicylaldoxime can discriminate between these components is still to be investigated.

The mechanism by which salicylaldoxime inhibits immune haemolysis is not resolved. Some information is afforded by the studies carried out on the transformation of cells in the state $EAC'_{1,4,2}$ to an inactive product, the decay reaction. As brought out by the results of Experiment VI, with a given reagent composed of arbitrary concentrations of C'_3 and the inhibitor, one can obtain a second reagent consisting of a less concentrated solution of C'_3 alone that will give the equivalent haemolytic effect, as measured by the total amount of lysis of a preparation of $EAC'_{1,4,2}$. Not only are the two reagents alike when measured statically, in terms of their total lytic capacity, but they appear to be equivalent kinetically, as shown by their effect on the rate of decay of $EAC'_{1,4,2}$. These results suggest that the effect of the inhibitor on haemolysis is similar to that produced by a diminished concentration of C'_3. This cannot be ascribed to the irreversible destruction of one of the components of the haemolytic system, since incubation of EA, or $EAC'_{1,4,2}$ or complement (measured both in $C'H_{50}$ and C'_3 units) with salicylaldoxime, followed by elimination of the inhibitor, causes no significant loss of haemolytic activity.

Since, for activity, the presence of the inhibitor during the course of the reaction appears to be mandatory, it is possible that the inhibitor competes for, or blocks, a site necessary for the lytic combination of $EAC'_{1,4,2}$ and C'_3. One possibility is that the combination of $EAC'_{1,4,2}$ and C'_3 activates an enzyme, perhaps C'_3 or one of the other complement components, or a proenzyme on the red cell. The substrate of this enzyme would presumably be on the cell surface, and the inhibitor could act by combining with the substrate, or with a site sufficiently near the substrate, to block enzymatic activity. If this mechanism were true, then it is of interest that this hypothetical substrate is not restricted to the sheep red cell. Preliminary experiments have shown that salicylaldoxime inhibits other complement-dependent reactions including the neutralization of T_2 phage by normal human serum, immune adherence, and the conversion of certain Gram-negative bacteria such as *Salmonella typhosa* to protoplasts.

Other explanations for the mechanism of salicylaldoxime action are, however, equally admissible. For example, the site at which the inhibitor acts may not be on the red cell but on one of the other components of the haemolytic system. In addition, the inhibitor

might act by preventing the activation of the hypothetical enzyme, or by preventing the actual combination between C'_3 and $EAC'_{1,4,2}$. The latter possibility is under study by examination of the effect of the inhibitor on the fixation of C'_3 by sheep red cell stromata–anti-stromata and by soluble antigen–antibody systems.

Unfortunately, there is as yet no significant information on the nature of the substance with which the inhibitor reacts. From the results obtained with *m*-hydroxybenzaldehyde, indicating that it inhibits by a mechanism similar to that of salicylaldoxime, it appears unlikely that a metal is involved. Chelation by *m*-hydroxybenzaldehyde would necessitate the formation of a seven-membered ring, and this is a rare event, particularly in aqueous solution (Martell and Calvin, 1952). The experiments with the compounds structurally related to salicylaldoxime must be interpreted with caution, since they are fragmentary and, with the exception of *m*-hydroxybenzaldehyde, it was not determined whether these compounds inhibit as does salicylaldoxime. However, if there is a common mechanism of inhibition, then it may be that a lipid component of the haemolytic system is involved. The evidence for this is slight and comes from the fact (Table 4) that inhibition tends to decrease with the introduction of a negative charge, as in salicylic or benzoic acid, or with substitutions on the benzene ring that serve to increase the aqueous solubility of the compound, as in the series *o*-, *m*-, and *p*-hydroxybenzaldehyde. Of the compounds thus far examined the most potent inhibitors are the ortho-substituted phenols. It is of interest that Rodriquez and Osler (1958 a,b) working independently with another ortho-substituted phenol, phlorizin, have obtained results that in many respects parallel those reported here.

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