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MEMBRANE MODIFICATION THROUGH THE COOPERATIVE ACTION OF VITAMIN A AND LIPID

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SUMMARY

Sheep red blood cells differ from the erythrocytes of several other species in that they are not lysed by simple exposure to vitamin A aldehyde (retinal). Lysis will occur however if the retinal treatment is followed by addition of lecithin. Lysis does not take place unless lecithin is added during a time period extending from 3 to 7 min after the retinal treatment is initiated; lecithin introduced earlier or later fails to disrupt the erythrocytes. Lysis mediated by the retinal–lecithin sequence is prevented if the red cells receive a preliminary treatment with retinal.

Dingle and Lucy [1] found that the red cells of rabbit, pig, ox, rat and man were rapidly lysed by vitamin A alcohol (retinol). It was suggested that the initial action of the vitamin on the red cell is penetration and expansion of the membrane; subsequent breaks in the membrane result in the loss of haemoglobin. In the present report we describe our findings relative to the lysis of sheep red cells through the cooperative action of vitamin A aldehyde (retinal) and certain lipids.

It was found that the red blood cells of guinea pig, rabbit, rat and human were lysed by treatment with retinal; sheep erythrocytes were an exception in that they were not lysed by retinal at any of the concentrations tested. But retinal lysis of sheep red cells was possible if the vitamin A treatment was followed by exposure to certain lipid substances; in order to obtain lysis, the lipid treatment must take place within a certain time period after the retinal addition. The solid line in Fig. 1 illustrates this temporal constraint: at zero time retinal was added to a suspension of sheep erythrocytes; at various times thereafter (minutes preincubation) an aliquot was removed and added to lecithin; the resulting mixture was then incubated an additional 20 min at 37 °C. Fig. 1 reveals that if the lecithin treatment is initiated too early, the red cell retinal mixture is not ready for lysis; and that if lecithin treatment is begun too late, the potentially lytic state has decayed. The optimum time for sheep erythrocyte–retinal interaction (at 30 °C) was found to be 3 min. It is possible to retard the decay of the potentially lytic state by lowering the temperature. This is illustrated by the broken line in Fig. 1. Retinal and sheep red cells were mixed at 30 °C as before, but at 3 min the red cell–retinal mixture was transferred to an ice bath and quenched with an equal volume of 0 °C buffer.

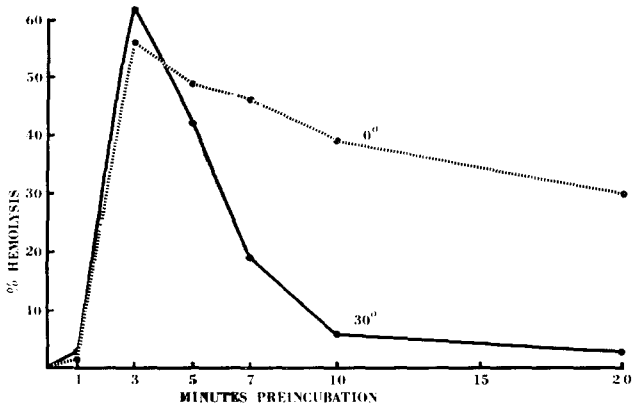

Fig. 1. Time course of haemolysis of sheep red cells by retinal–lecithin treatment. 30 ml of erythrocytes ($1.5 \cdot 10^8$ cells/ml) were treated with 3.0 ml of retinal ($4 \cdot 10^{-5}$ M) at 30 °C; this treatment is referred to as preincubation. At 1 min 0.5 ml of the preincubation mixture was removed to 2.5 ml of lecithin. At 3 min, 5.5 ml of preincubation mixture was added to an equal volume of 0 °C buffer and transferred to an ice bath. A second 5.5-ml aliquot of preincubation mixture was added to an equal volume of 30 °C buffer and maintained at that temperature. At various times 1.0-ml samples were removed from each series and added to 2.0 ml of lecithin. The retinal–erythrocyte lecithin mixtures were then incubated an additional 20 min at 37 °C. The final reaction mixture of 3.0 ml contained $3.7 \cdot 10^7$ red cells, $1 \cdot 10^{-8}$ moles retinal and $3.3 \cdot 10^{-8}$ moles lecithin. The extent of lysis was estimated by centrifuging the mixtures and determining the absorbance of the supernatant fluids at 412 nm; controls for 100% lysis and cell fragility were included. The experiments described in this report were carried out in veronal buffered saline containing 0.01 M EDTA [2]. Sheep erythrocytes (high K^+ [3]) were collected as described by Mayer [4]; before use the erythrocytes were washed 3 times with buffer. All-*trans* retinal was obtained from Distillation Products Industries and L-α-lecithin (beef, chromatographically pure) from General Biochemicals; these compounds were used without further purification. Stock solutions of retinal and lecithin were maintained in absolute ethanol and diluted in buffer for use. The diluted retinal was slightly opalescent; it was used immediately after dilution to avoid oxidation. At its final dilution in buffer lecithin appeared to be in solution.

Thereafter samples were removed and treated with lecithin as usual. From Fig. 1 we might infer that after retinal has "penetrated" the membrane, reduction of the temperature to 0 °C delays the decay of the potentially lytic state. It is significant that the first 3 min of preincubation must be carried out at an elevated temperature (e.g. 30 °C); if the entire preincubation with retinal is conducted at 0 °C, no lysis takes place on transferring the cells to lecithin. Presumably the initial penetration, as well as the decay, is depressed at low temperatures. The basic findings reported here (solid line, Fig. 1) were obtained with erythrocytes from 3 sheep at West Virginia University and one sheep at another institution.

During the course of this work it was discovered that if sheep red cells were treated with retinal and allowed to decay, retreatment with retinal followed by lecithin did not result in lysis; apparently retinal pre-treatment altered the cell membrane in such a way that it was no longer vulnerable to disruption by a subsequent retinal–lecithin treatment. A dose–response study of these 2 activities of retinal revealed an interesting relationship (Fig. 2). The lysis of sheep red cells by retinal and lecithin (broken line, Fig. 2) displays a lag relative to retinal concentration On the other hand there is no evidence of such a lag in the dose response of retinal

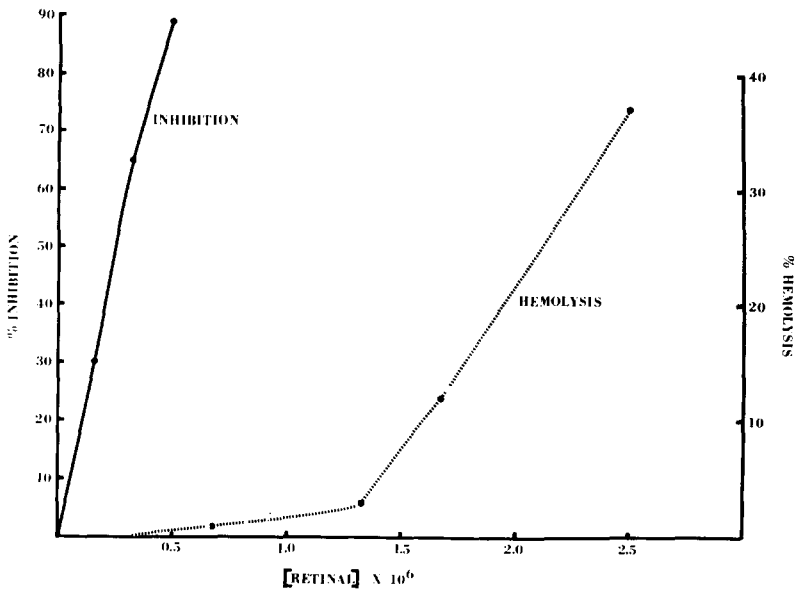

Fig. 2. Influence of retinal concentration (molarity) on (a) haemolysis caused by retinal–lecithin treatment (●···●) and (b) retinal inhibition of retinal–lecithin haemolysis (●—●). (a) Haemolysis: $3.7 \cdot 10^7$ sheep erythrocytes were treated with retinal in a volume of 1.0 ml for 3 min at 30 °C; 2.0 ml of $3.1 \cdot 10^{-5}$ M lecithin were then added and the mixture incubated at 37 °C for 30 min. (b) Inhibition of retinal–lecithin haemolysis: $3.7 \cdot 10^7$ sheep erythrocytes were treated with retinal in a volume of 1.0 ml for 15 min at 30 °C; the mixture was centrifuged and the supernatant fluids discarded; the cells were resuspended in 1.0 ml of $1 \cdot 10^{-5}$ M retinal and held at 30 °C for 3 min; 2.0 ml of $3.1 \cdot 10^{-5}$ M lecithin were added and the tubes incubated at 37 °C for 30 min.

inhibition (solid line, Fig. 2). The qualitative difference in the curves might be accounted for in the following way: the lag in the lytic response to the dose of retinal suggests that a particular membrane site would be vulnerable to disruption by lecithin only if more than one molecule of retinal had interacted with that site; at the very low concentrations of retinal, most sites would not have accumulated a sufficient number of retinal molecules to be disrupted by lecithin. Conversely, the absence of a lag in the inhibition curve implies that only a single retinal molecule need penetrate a site in order to prevent lysis by subsequent retinal–lecithin treatment. It is apparent from Fig. 2 that much smaller quantities of retinal are needed to inhibit lysis than are necessary to mediate lysis. We were also able to prevent retinal–lecithin lysis of sheep cells by pretreating the lecithin with retinal. In a typical experiment, a lecithin suspension at a concentration of $6.2 \cdot 10^{-5}$ M was treated with an equal volume of $4 \cdot 10^{-5}$ M retinal. When 2.5 ml of this mixture was added to 0.5 ml of a sheep red cell suspension which had been treated with retinal 3 min previously, no lysis occurred. The experimental design was patterned after the one illustrated in Fig. 1. The control experiment involving untreated lecithin resulted in lysis.

When the lecithin lysis of retinal-sensitized sheep cells was examined as a function of retinal concentration, the shapes of the dose response curves were unexpected (solid lines, Fig. 3) in that they increased to a maximum and then dropped

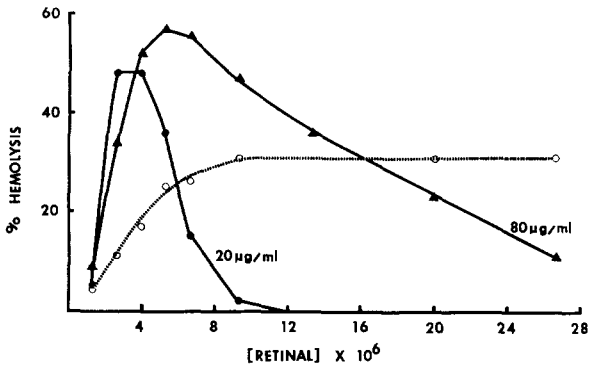

Fig. 3. Influence of retinal concentration (molarity) on the lecithin lysis of retinal-sensitized sheep erythrocytes. Solid lines: $7.5 \cdot 10^7$ sheep erythrocytes were treated with various concentrations of retinal in a total volume of 2.5 ml for 3 min at 30 °C; 3.5 ml of lecithin at concentrations of 20 µg/ml and 80 µg/ml ($2.5 \cdot 10^{-5}$ M and $10 \cdot 10^{-5}$ M, respectively) were then added. The cells were incubated 20 min at 37 °C. The percentage of cells surviving was determined by centrifuging the mixtures, draining the supernatant fluids and lysing the surviving cells with 6.0 ml distilled water. The percent haemolysis was calculated from the absorbance of these solutions at 412 nm. Broken line: $7.5 \cdot 10^7$ sheep erythrocytes were treated with various concentrations of retinal in a total volume of 2.5 ml. After 3 min at 30 °C the tubes were chilled, centrifuged and drained. The packed cells were resuspended in 3.5 ml of lecithin ($2.5 \cdot 10^{-5}$ M) and 2.5 ml buffer and incubated 20 min at 37 °C after which the percent haemolysis was determined.

off. In keeping with the experiment just described, one might suggest that at higher retinal concentrations there is an excess of this substance in the fluid phase where it can react with the added lecithin and thus prevent lysis. This experiment was carried out using 2 concentrations of lecithin and the shapes of the 2 curves are consistent with this explanation; the dose response curve is displaced to the right by increasing the concentration of lecithin, which indicates that more retinal is required in the fluid phase to "saturate" the lecithin. This suggestion was tested by treating sheep red cells with appropriate quantities of retinal and then removing the fluid phase retinal by centrifugation. When cells thus sensitized were exposed to lecithin, they lysed as shown by the broken line in Fig. 3; in the absence of fluid phase retinal the extent of haemolysis was not depressed at the higher concentrations of this substance. The degree of lysis in the latter experiment (broken line, Fig. 3) is less than in the other two shown in that figure because the time needed for the centrifugation was greater than 3 min and thus the sensitized state had already begun to decay (see solid line, Fig. 1).

The events described here are consistent with the following model. The molecules of vitamin A aldehyde penetrate the sheep red cell membrane without causing disruption. If lecithin (or certain other lipids) is available for interaction with the membrane associated retinal, breakage and haemoglobin release will result. But if too much time is allowed to pass before adding lecithin, the retinal-sensitized state will decay; this might be due to further penetration into the membrane with the result that the retinal molecules are no longer accessible to lecithin.

In addition to lecithin, a number of other materials could be used to lyse retinal-sensitized sheep cells. These included olive oil, cholesterol, α-tocopherol

and retinyl acetate, plus methanol–chloroform extracts of guinea pig serum and bovine serum albumin. These materials share the property of lipid solubility. It came as no surprise that glycerol did not lyse retinal-sensitized cells. The lysis of vitamin A-sensitized cells by an accessory agent was previously reported by Dingle and Lucy [1]. These workers found that when rabbit red cells were treated with vitamin A alcohol (retinol) at temperatures below 20 °C, lysis was slowed or prevented; these intact vitamin A-treated cells displayed an altered reactivity in that they could be rapidly and completely lysed by the addition of serum or bovine serum albumin. Albumin which had been "saturated" with retinol was inactive. Dingle and Lucy [1] suggested that the added protein caused lysis by combining with the molecules of vitamin A present in the membrane. Our findings with lecithin and other lipids suggested that the results of Dingle and Lucy were due not to the protein which they were adding but to an associated lipid. This possibility was tested by subjecting rabbit serum and bovine serum albumin solution to chloroform–methanol extraction [5]; when either of these extracted materials was added to retinol sensitized rabbit erythrocytes at 14 °C (the cell preparation used by Dingle and Lucy), rapid lysis ensued. We also observed that such rabbit cells can be lysed by lecithin.

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