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THE INFLUENCE OF RETINAL ON COMPLEMENT-DEPENDENT IMMUNE DAMAGE TO LIPOSOMES

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SUMMARY

Retinal was incorporated into liposomes containing dipalmitoyllecithin, cholesterol, dicetyl phosphate and galactocerebroside; the latter substance served as antigen. They were compared to control liposomes, lacking retinal, with regard to glucose release due to complement-dependent immune damage in the presence of anticerebroside serum. The liposomes were indistinguishable from each other in the amount of total glucose trapped, light scattering characteristics and phosphate content. The rate and extent of glucose release in 30 min was inhibited by the incorporation of retinal. In addition, inhibition was directly related to retinal concentration and was also observed in the presence of a wide range of concentrations of antigen and complement. Damage to liposomes in the presence of either guinea pig or human complement was inhibited by retinal; this was in contrast to the erythrocyte system in which the hemolytic activity of guinea pig complement was inhibited while that of human complement was enhanced by retinal. Addition of retinal to preformed liposomes did not influence complement-dependent damage. Inhibition occurred only when retinal was present during the initial formation of the model membranes. Inhibition persisted even after washing the liposomes free of any unincorporated retinal. The data indicate that liposomes may be an excellent model for studying the influence of retinal on complement mechanism in membranes.

INTRODUCTION

Previous studies have demonstrated that liposomes may serve as a useful alternative to whole cells for investigating the molecular mechanism of complement-dependent immune lysis (recent reviews in refs 1 and 2). Many remarkable similarities between liposomes and cells have been observed in regard to the effects of antigen, antibodies and complement on membrane damage. Because of their simplicity, however, liposomes have a unique advantage over cells in that they are protein-free, and the individual constituents can be altered in regard to lipid

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type and concentration. Therefore, it has been possible to investigate the influence of phospholipid and fatty acid compositions [3,4], surface charge [3] and antigen type and concentration [3,5–8] on immune damage to bilayer membranes. The major conclusion drawn from this approach has been that most, and probably all, of the sequential steps involved in complement-dependent immune damage can be reproduced with a completely synthetic lipid membrane system.

The process of immune damage by complement has been shown to be influenced by differences in membrane constituents. Vitamin A (retinol, retinal and retinoic acid) is one "membrane-soluble" substance which has been shown to exert an effect on the immune hemolysis of sheep erythrocytes. This effect was first reported by Billitteri and Raoul [9]. They made the observation that retinal inhibited the guinea pig complement-dependent lysis of sensitized sheep erythrocytes. This initial work was confirmed and greatly extended by Major et al. [10] who found that, with guinea pig complement, retinol and retinal were about equally effective as inhibitors of immune lysis. Retinoic acid was about half as active with respect to the inhibition and in a later report [11] β-ionone, a short chain analogue of vitamin A, was also found to be inhibitory. Retinal was used for most of their work because, unlike retinol, it was not hemolytic at concentrations where considerable inhibition of immune lysis was observed. It was found [10] that several different cellular intermediates (interrupted at various stages in the complement "cascade") were susceptible to inhibition by micromolar concentrations of retinal. This inhibitory property of retinal persisted even after extensive washing and because of this it was suggested that retinal interacted with, and was probably adsorbed onto the membrane of the red cell. In contrast to the inhibition due to the interaction of vitamin A derivatives with guinea pig complement, retinal was shown to have an enhancing effect on the immune hemolysis mediated by human complement [12]. The reason for this species difference is unknown; however, it seems reasonable to assume that in this case also, retinal entered a crucial lipid area of the membrane and by so doing profoundly altered the complement reaction.

This paper is aimed at further delineating vitamin A–membrane–complement interaction by studying the effect of retinal on the complement-mediated damage to liposomes. Retinal was incorporated into immunologically responsive liposomes containing a mixture of natural and synthetic lipids and was found to be inhibitory. The relative influences of antigen and complement on the inhibitory effect of retinal were investigated.

MATERIALS AND METHODS*

Enzymes and cofactors

Glucose-6-phosphate dehydrogenase and hexokinase were obtained from Boehringer-Mannheim Corp., New York, N.Y. ATP was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. NADP was from Calbiochem., Los Angeles, Calif.

* In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council.

Lipids

The lipids came from the following companies: synthetic β,γ-dipalmitoylphosphatidylcholine (lecithin) and cholesterol (Calbiochem., Los Angeles, Calif.); dicetyl phosphate (K and K Laboratories, Inc., Plainview, N.Y.); all-*trans* retinal (Eastman Kodak Co., Rochester, N.Y.). Beef brain galactocerebroside which was incorporated into liposomes came from Schwartz/Mann, Orangeburg, N.Y., galactocerebroside used for immunization was from Applied Science Laboratories, State College, Pa. Retinal (10 mM) was dissolved in ethanol, stored under nitrogen at -20 °C and was used within a month.

Preparation of liposomes and assays of trapping and glucose release

Liposomes were made on a daily basis from mixtures of dipalmitoyllecithin, cholesterol, and dicetyl phosphate which were present in molar ratios of 2:1.5:0.22, respectively. With respect to the final aqueous suspension of swollen liposomes, the lipids were in concentrations of 10 mM, 7.5 mM and 1.1 mM, respectively. Except where indicated, cerebroside was present as 150 μg/μmole of phospholipid. Retinal, when initially present in the liposomes, was added in ethanol to the chloroform solution before the initial drying down of the lipids (see ref. 3 for complete details of liposomes preparation). In all cases, the retinal concentration is described in terms of millimolarity; this refers to the concentration in the aqueous suspension of liposomes. The dried lipids were swollen in 0.308 M glucose and dialyzed for 90 min against 1250 vol. of 0.154 M NaCl.

Total trapped glucose was measured by the "chloroform–Triton" method previously described [3] (see ref. 13 for correction of a typographical error). The Tris-buffered glucose assay method was identical to that previously described [3] except that 0.154 M NaCl was substituted for veronal buffer and half the amounts of glucose-6-phosphate dehydrogenase and hexokinase were present.

Measurement of release of trapped glucose due to the immunological reaction was performed essentially by methods previously described [3]. Because of anticomplementary activity in the anticerebroside serum, assays were always started by adding antiserum (or complement) last. Due to the rapidity of the reaction kinetics, as shown below, any effect of anticomplementary activity was minimized by this procedure.

Fresh serum

Human serum was obtained by venipuncture and was not pooled. Pooled guinea pig serum was obtained by terminal bleeding without anaesthesia. The sera were dialyzed extensively against cold 0.154 M NaCl to reduce the levels of glucose and were then frozen at -70 °C until needed.

Preparation of anticerebroside sera

Anticerebroside serum from rabbits was produced by a slight modification of the method of Niedieck et al. [14]. Each animal was immunized with 1 ml of an emulsion consisting of 7 mg cerebroside, 2.5 mg bovine serum albumin in 0.5 ml of 0.154 M NaCl and 0.5 ml Freund's complete adjuvant. Injections were made into all foot pads and one subcutaneous site. Serum was obtained by cardiac puncture after either two or three weeks and was heated routinely at 56 °C for one hour

before use. Sera obtained from different animals were not pooled. The level of anticerebroside activity after two weeks was comparable to that reported following injections of beef brain after much longer intervals [6]. Although very early bleedings were always used, it was observed that the titer remained relatively high for as long as three months or more after a single immunization. This method of immunization with a purified antigen had the advantage that it eliminated the necessity of injecting beef brain and avoided the complications which were reported to be associated with that technique [6]. When IgM and IgG fractions isolated on Sephadex G-200 were compared, the activity of antisera after three weeks could be attributed entirely to the fraction containing IgM.

RESULTS

Inhibitory effect of retinal on imune-specific glucose release

In order to demonstrate inhibition due to retinal, liposomes were constructed which contained synthetic dipalmitoyllecithin, cholesterol, dicetyl phosphate and cerebroside and which either contained or lacked retinal. The time course of glucose release from these liposomes in the presence of specific antiserum and guinea pig complement is shown in Fig. 1. This experiment demonstrates that, with regard to the kinetics of glucose release, the initial lag was increased and both the rate of glucose release and the final plateau were decreased with the retinal-containing liposomes as compared to those which lacked retinal. It should be pointed out that the immunochemical reaction was probably completed within 10 min in both cases. Despite this some glucose continued to diffuse out of the liposomes, and the rate

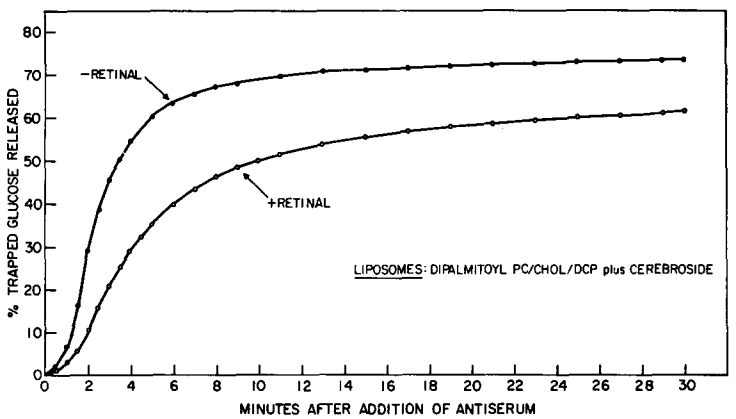

Fig. 1. Effect of retinal on the time course of immune damage to liposomes. The liposomes contained sufficient retinal to make a final concentration of 1.0 mM in the swollen suspension. 4.8 μl of liposomes were preincubated for 10 min with 128 μl of fresh guinea pig serum (complement), 500 μl of glucose assay reagent and sufficient 0.154 M NaCl to make a volume of 976 μl in a semi-micro cuvette. After reading the absorbance at 340 nm the immunological reaction was started by adding 24 μl of rabbit anticerebroside serum. The final absorbance readings were measured at the time intervals shown and the % of trapped glucose released was determined from the difference compared to the initial reading. Less than 4% of the available trapped glucose was released in 30 min in control cuvettes in which complement had either been omitted or inactivated by heating at 56 °C for 60 min.

of this terminal diffusion was more rapid from the retinal-containing liposomes. Because of this, the apparent degree of inhibition, which can be calculated from the data shown in Fig. 1, decreased from 28% at 10 min. to 17% at 30 min. Subsequent measurements were obtained at 30 min., however, in order to conform to previously published assay techniques [3]. The rabbit antiserum and fresh guinea pig serum were both present in excess quantities. As observed in other studies [15,3], 100% glucose release was not observed after 30 min under optimum conditions with either liposome preparation. The reason for this is not completely understood.

Effect of retinal and complement concentrations on inhibition

In Fig. 2 an experiment is shown in which liposomes were prepared containing different concentrations of retinal. The glucose release in each case is illustrated as a function of guinea pig serum concentration, and it was found that the amount of complement-dependent glucose release was inversely related to retinal concentration. The most prominent feature of the inhibition was again that the upper limit of glucose release after 30 min (i.e. the plateau) was consistently diminished. When the concentration of retinal was further increased the liposomes gradually became more unstable, and at higher levels (approx. 1.5 mM) they nonspecifically released large amounts of trapped glucose after 30 min. in the presence of either fresh or heated serum. Because of this, retinal concentrations above 1.0 mM were never used. The alcohol form of vitamin A (retinol) was also assayed and found to be inhibitory to the immune response. It was not used routinely, however, because it caused the liposomes to be more unstable.

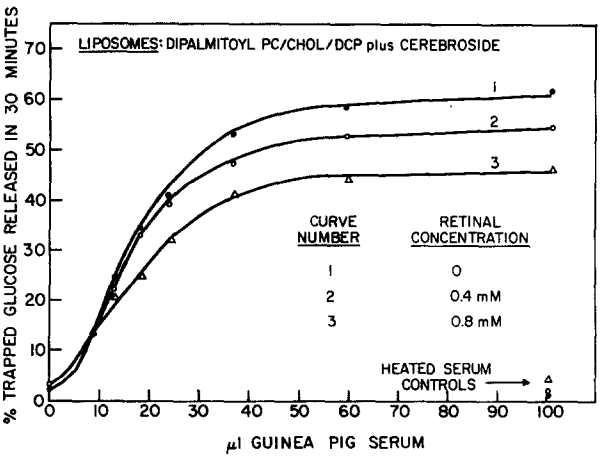

Fig. 2. Effect of retinal concentration on immune damage to liposomes. The procedure was the same as described in the legend of Fig. 1. Retinal was incorporated directly into the liposomes as described in Materials and Methods, and was present in the concentrations, with respect to the swollen dispersions, indicated in the figure.

Effect of antigen on retinal inhibition

Liposomes are unique in that, detailed experiments can be performed involving different concentrations and types of antigens in the same lipid matrix. Extensive experiments [3] have demonstrated the importance of antigen concentration in

immune-specific glucose release from liposomes. Variation of the amount of antigen is a method of altering the number of antigen–antibody sites which, in turn, regulates the degree of damage due to complement. In Fig. 3 the depression of glucose release due to retinal is plotted as a function of antigen concentration, and clearcut inhibition of immune-specific glucose release was observed at every level. In this figure every antigen concentration represents a separate liposome preparation. The maximal amount of glucose released (the plateau) varied somewhat from day to day, and this variability was partly a function of the method of daily liposome preparation. Because of this, the figure shown is a composite illustration, and the points illustrated were averages taken from several experiments. At the highest concentration of antigen (150 μg cerebroside/μmole phospholipid) $n=6$ for liposomes containing retinal and $n=7$ for liposomes lacking retinal. The bars represent one standard deviation away from the mean and the differences shown are highly significant ($P<0.005$).

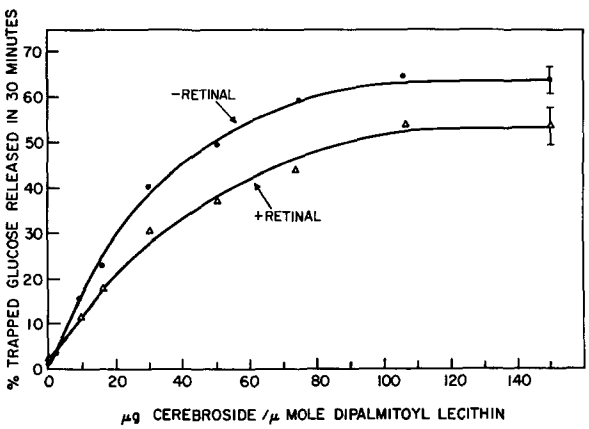

Fig. 3. Retinal inhibition of glucose release from liposomes containing various concentrations of antigen. Liposomes were constructed from dipalmitoyl-lecithin, cholesterol, dicetyl phosphate and cerebroside; the amounts of cerebroside are indicated on the abscissa. Retinal, where present, was 1.0 mM with respect to the swollen liposomes. The assay procedure was the same as described in the legend of Fig. 1. The bars represent one standard deviation in each direction from the mean (see text for further details).

Human complement

The inhibitory effects of retinal shown above were all observed with guinea pig serum as a complement source. These results are in agreement with conclusions from the previous work [10] in which the immune hemolysis of sheep erythrocytes due to guinea pig serum was inhibited by retinal. As also stated above, however, retinal had an enhancing effect on hemolysis when human serum was used as a complement source [12]. Because of this the effect of retinal was investigated with liposomes and human complement. In Fig. 4 an experiment is shown in which liposomes either containing or lacking retinal were incubated with antiserum and with human serum as the complement source. Inhibition of glucose release was again observed.

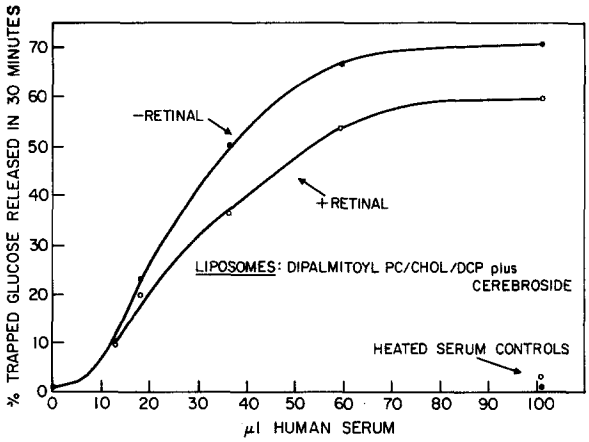

Fig. 4. Effect of retinal on immune damage by human complement. The liposomes and assay conditions were the same as in the legend of Fig. 1 except that human serum was used as the complement source. Retinal, where present, was in the concentration of 1.0 mM with respect to the swollen liposomes.

Inhibition by "passive" addition of retinal

In the experiments shown above, retinal was added before the initial drying of the lipids (i.e. before the liposomes were made). In the experiment shown in Fig. 5 retinal, in ethanol, was added to the assay cuvette before the immune reaction. Retinal which was added to the "fluid phase" in this manner did not inhibit the immune response of liposomes at a concentration (1 mM) which was equivalent to

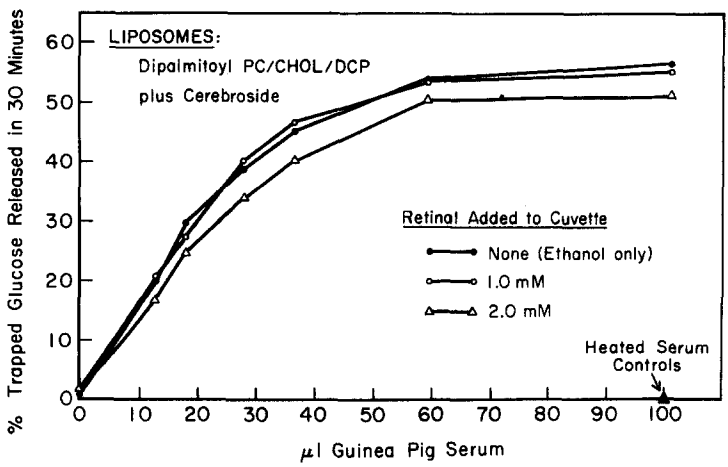

Fig. 5. The effect of exogenous retinal on the immune response of liposomes. The assay procedure was essentially the same as described in the legend of Fig. 1. The liposomes were prepared in the absence of retinal. During the preincubation period with guinea pig serum retinal was also present, where indicated. For convenience, the amounts of retinal refer, as before, to the concentration relative to the volume of liposomes present. The retinal (0.01 M in ethanol) was diluted in 0.154 M saline to 10^{-4} M and then added to the cuvettes to give the concentrations indicated. The control liposomes were treated with an identical quantity of ethanol.

the highest amount used in the previous experiments. At twice this concentration (2 mM) a slight degree of inhibition was observed. The heated serum controls demonstrated that this latter concentration of retinal did not affect liposome stability when added to the fluid phase. It was concluded from this experiment that addition of retinal by this method could cause some inhibition, but was much less efficient in doing so.

Removal of "unincorporated" retinal

Because retinal is a water-insoluble, amphipathic lipid, it was assumed that at least some of it was incorporated into the liposomal model membrane. This assumption was valid because the liposomes were obviously yellow, due to retinal, even after agglutination by specific antiserum. To further eliminate the possible influence of "fluid phase" retinal, liposomes were washed free of unincorporated retinal by differential centrifugation in the presence of specific antiserum. At a very low speed (approx. $1000 \times g$) liposomes containing antigen were quantitatively separated from any unincorporated retinal. Control experiments, which are not shown, demonstrated that less than 8% of micellar retinal was sedimented by this method, and two washes were sufficient to remove virtually all unincorporated retinal. Fig. 6 shows that even after these liposomes were washed free of unattached retinal, they were still subsequently inhibited in the immune response in the presence of guinea pig complement. As with the liposomes in the experiment shown in Fig. 5, however, significant inhibition of these washed liposomes was not observed when 1 mM retinal was added after the initial swelling of the liposomes.

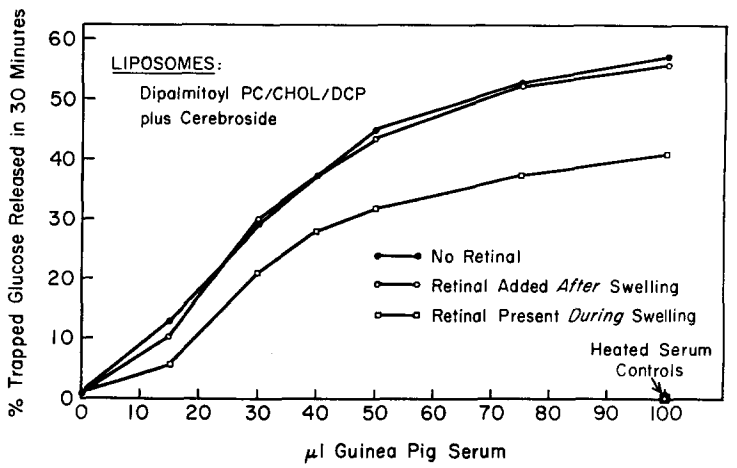

Fig. 6. Inhibition of glucose release from liposomes washed free of unincorporated retinal. 150 μl of liposomes which initially either lacked retinal (●—●, ○—○) or contained retinal (□—□) were incubated for 15 min with 150 μl of rabbit anticerebroside serum. 9 ml of 0.154 M NaCl and, where indicated (○—○), 15 μl of 10^{-2} M retinal in ethanol were then added and incubated for 30 min. The liposomes were separated from any unincorporated micellar retinal (i.e. washed) by centrifuging at $1000 \times g$ for 10 min at 20 °C. The liposome pellets were washed again in 9.5 ml of 0.154 M NaCl and were then resuspended in 1.0 ml. 31.8 μl of liposomes were preincubated with 10 μl of rabbit anticerebroside serum in assay cuvettes and the reaction was started by adding the amounts of guinea pig serum shown.

Geometrical aspects of the liposomes

In order to determine if the phenomena reported here were due to retinal-induced changes in liposomal geometry, several parameters were used to estimate the influence of retinal on the physical characteristics of liposomes. Table I shows that the amount of liposomal phospholipid recoverable by centrifugation was identical with the different liposome preparations. Furthermore, retinal did not cause any appreciable effects on the amount of total glucose trapped nor on the light scattering characteristics of liposomes. These observations indicate that retinal did not cause an obvious alteration in the geometrical characteristics of the liposomes.

TABLE I

COMPARISON OF PHYSICAL CHARACTERISTICS OF LIPOSOMES

Where indicated, retinal was present in 1.0 mM concentration. The data were adjusted to correspond to the amounts present in 5 μl of liposomes. Liposomal phosphate was measured in a washed pellet of liposomes which was obtained by centrifugation after adding specific antiserum. The absorbance due to light scattering of retinal-containing liposomes was adjusted for absorbance due to retinal at 340 nm. The differences shown between the liposomes in regard to light scattering and total glucose trapped were not statistically significant. The numbers in brackets are the standard deviations. See the text for further details.

Liposome composition	Total glucose trapped (nmoles)	Absorbance due to light scattering (340 nm)	Liposomal phosphate recovered (nmoles)
Dipalmitoylphosphatidylcholine/cholesterol/ dicetyl phosphate plus cerebroside	263 ± 31 *	0.113 ± 0.012 *	5.5 ***
Dipalmitolphosphatidylcholine/cholesterol/ dicetyl phosphate plus cerebroside plus retinal	276 ± 37 **	0.121 ± 0.011 **	5.5 ***

* Average of eighteen experiments.
** Average of eight experiments.
*** Average of two experiments.

DISCUSSION

Liposomes have proven to be a useful model for investigations into the nature of the membrane "substrate" for complement. Work with this system has demonstrated that immune damage can be influenced by differences in membrane constituents. Different immune susceptibilities have been found depending upon the composition of the liposomes [3,6,7].

The experiments described in this paper demonstrate that incorporation of retinal into liposomes also influenced their sensitivity to immune damage. It was shown (Fig. 1) that the rate and final extent of marker release were decreased in retinal-containing liposomes. The inhibition of glucose release was directly related to the retinal concentration (Fig. 2), and the occurrence of inhibition was not dependent on antigen concentration (Fig. 3). Vitamin A can thus be said to influence complement-dependent damage in both erythrocytes [9,10,12,16] and

liposomes. With regard to guinea pig serum as the complement source, retinal caused inhibition of immune damage in both systems [10,16]. It was also observed that, as with erythrocytes [17], high doses of retinal (and retinol) caused the liposomes to be quite unstable and release significant quantities of trapped glucose.

Several differences were noted in this study, however, between the effects of retinal on liposomes and previous observations of the influences of retinal on immune hemolysis. Incorporation of retinal into liposomes resulted in inhibition of glucose release in all cases regardless of the species used as a complement source. With erythrocytes, on the other hand, retinal had opposing effects; the hemolytic activity of guinea pig complement was inhibited [10,16], whereas with human complement the hemolytic activity was enhanced [12]. The reason for this species difference with erythrocytes has never been explained; nor is it clear why, in the present investigation, similar differences were not detected with liposomes. It is known that the hemolytic complement activity of guinea pig serum is about 6-fold greater than that of human serum [18]; in contrast, large differences were not observed between the complement titers of human and guinea pig sera when they were assayed with liposomes [19]. This was probably due to different rate-limiting steps in the two systems [19] and the same reasoning may, in part, explain why retinal caused inhibition with both sera in the liposomal system.

A further important difference of the liposome system in comparison with erythrocytes was in the method of addition of the retinal. With erythrocytes, the retinal was, of necessity, added to the pre-existing membrane. In contrast, addition of 1 mM retinal to the liposomes after they had been made did not result in a significant inhibition of immune-specific glucose release (Figs 5 and 6). Inhibition only occurred when the retinal was added prior to the formation of the liposomal membrane. Liposomes prepared by this method are concentric bilayers [20] and it seems quite possible that when retinal was added to the already swollen liposomes it was incorporated only into the outer surface. This might account for the lack of detectable inhibition. When retinal was added during the initial preparation of the liposomes, however, it would then have been equally dispersed through all the bilayers.

The mechanism by which vitamin A influences complement lysis is not known. In previous work with the erythrocyte system it was observed that retinal exerted an effect on the complement-mediated reaction even when it was added after all the complement components had interacted with the red cells [10]. This observation indicated that at least part of the effect of retinal was due to an interaction with membrane-associated reactions. The mechanism of the retinal effect on immune damage to liposomes is also not clear. The present work is compatible with the suggestion that retinal inhibited the immune damage by a mechanism other than by a direct interaction with fluid phase complement. Liposomes which were washed free of unattached retinal were still inhibited with respect to immune-specific glucose release (Fig. 6). Inhibition occurred, but was much less effective if the retinal was exposed only to the outer surface of the liposomes (Fig. 5). This work supports the previous hypothesis [10] that retinal may exert its effect on erythrocytes, and perhaps other cell membranes, by entering the lipid portion of the membrane which serves as "substrate" for complement damage.

Apparent changes in the physical structure of liposomes due to incorporation

of retinol have been previously reported based on a study utilizing the electron microscope [21]. Significant alterations of liposomal geometry were not observed in the work reported here using the parameters shown in Table I. This possibility has not necessarily been completely eliminated in the present work with retinal, however, and this is still under investigation with other techniques.

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