HOMOLOGOUS IMMUNOLOGICAL STUDIES OF OCULAR LENS*

I. In Vitro Observations

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Plates 31 and 32

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Ocular lens has long been known to contain antigens capable of eliciting an antibody response in heterologous species (1–4). It was shown by Uhlenhuth (1) that an antiserum prepared against one mammalian lens would cross-react to a high degree with lens of other mammalian species, and to a more limited degree with bird or fish lenses. These findings were confirmed by a number of investigators (see reference 5). Hektoen and Schulhof (6) first studied the protein fractions responsible for those reactions, and demonstrated with rabbit antisera that beef lens alpha and beta crystallines were distinct immunological entities. They also demonstrated that such anti–beef lens sera did not cross-react with beef serum. These results were confirmed by Woods and Burky (3). In addition, Hektoen showed that the species' cross-reactivities of the beef lens fraction antisera (anti-alpha and anti-beta crystalline prepared in the rabbit) were caused by the antigenic similarity of these chemically isolated components in beef, rabbit, swine, monkey, and menhaden (fish) lens. Wollman *et al.* (7) carried the study of the organ specificity of lens a little further down in the evolutionary scale. They found that rabbit antisera against the lens of the *Octopus vulgaris* did *not* cross-react with mammalian lens, although it had a high titer against homologous antigen. Similarly no cross-reactivity was found between anti-mammalian lens sera and octopus lens. Practically all investigators who have studied this problem, therefore, agree that the antigenic specificities of lens substances are similar through a wide segment of the animal kingdom (organ specificity).

Although antisera to heterologous lenses can be readily produced in rabbits, and the antibodies thus produced will react to a high titer with rabbit lens, difficulty has been experienced in producing precipitating antibodies against rabbit lens in the rabbit (homologous). Thus Hektoen (2), Hektoen and Schulhof (6), and Woods, Burky, and Woodhall (8) did not find any detectable antibody response in rabbits to repeated doses of rabbit lens homogenate. The former investigators did find an occasional response in rabbits which had previously received injections of heterologous

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lens. Both groups detected precipitating antibodies in rabbits which had received repeated doses of alpha crystalline fraction, chemically isolated from *rabbit* lens. Whereas Hektoen and Schulhof (6) also found isolated *rabbit* beta crystalline to be antigenic in the rabbit, Woods *et al.* (8) failed to show antigenicity of this material or of rabbit gamma crystalline in the homologous species.

The development of new and powerful immunological technics in recent years has prompted a reinvestigation of the problems outlined. Stress has been placed here on homologous lens immunization (*e.g.* adult rabbit lens into rabbits) in order to avoid complications that could conceivably arise in heterologous immunizations, due to non-lens antibodies. Following the discoveries of Freund and his group (9–12) many reports have shown the value of his adjuvant mixture for the enhancement and prolongation of antibody responses. In addition, antibody responses to *homologous* brain (13, 14), testis (15), and uveal tract (16) with resulting immunological lesions may be obtained fairly quickly and readily with some species. The effectiveness of this adjuvant mixture in producing homologous rabbit lens antibodies in the rabbit will be reported here.

Most of the investigators who have studied this lens problem used simple precipitin titrations in small test tubes or in capillary tubes. Estimations of the relative potencies of various sera were usually made by serial dilution of the test antigen (2–4). This method is somewhat insensitive in general, and gives poor information regarding relative antibody content of several sera (17). In addition, these technics give no information about the number of antigen-antibody systems that may be involved in a given reaction. The agar precipitin methods which have recently been developed are highly sensitive to small amounts of antigen and antibody, and give clear and direct evidence of the number of immunologically distinct systems involved in a given reacting mixture. Several modifications of this basic idea have been worked out, notably by Oudin (18), Ouchterlony (19), Elek (20), and Oakley and Fulthorpe (21). The Ouchterlony approach has been found most suitable for this work with homologous lens antibodies. A recent report by Rao *et al.* (22) has utilized one of these methods (Oakley's) in the study of *heterologous* anti-lens sera (bovine lens injected into rabbits). These antisera showed six antigen-antibody systems when tested against bovine lens, two when tested with bovine vitreous, and none with bovine serum.

Although precipitin analysis in agar gives clear evidence of the multiplicity and identities of the antigen-antibody systems in a particular mixture, this method gives comparatively little quantitative information about the relative potencies of antisera in their behavior toward a given antigen. For this type of information, use has been made of the hemagglutination technic, (23, 24, 28) using lens in the adsorbing antigen mixture. The technic is extremely sensitive to small amounts of antibody, and is very useful for comparative estimations of

antibody content in different sera. By a hemagglutinin inhibition technic it can also be used to quantitate antigens. Adaptation to the study of lens antibodies has proven very simple and effective, as reported here.

In studies carried out by Woods, Burky, and Woodhall (8), subsequently confirmed by Swift and Schultz (25) and Marsh (4), it was shown that staphylococcal "toxin" enhances the antibody response against *heterologous* lens (*bovine* lens into rabbits). Swift and Schultz found that this toxin was unable to significantly stimulate the formation of precipitating *rabbit* lens antibodies in the rabbit under the conditions employed. The alpha toxin was presumed by the above workers to be the adjuvant agent. Because of many similarities between the biological activities of staphylococcal alpha toxin and beta hemolytic streptococcal oxygen-labile hemolysin (streptolysin"O"), a small number of rabbits were tested to see whether this latter material showed any adjuvant activity with *homologous* lens.

Materials and Methods

The lens pool used for immunization was obtained aseptically from freshly sacrificed adult rabbits (6 to 8 pounds) of various breeds. These were weighed wet, and homogenized to a concentration of 100 mg. wet weight/ml. in sterile 0.85 per cent NaCl, using a teflon-glass tissue grinder (A. H. Thomas, Philadelphia). A large enough pool was prepared to satisfy all the needs of the study reported below. This was stored in the deep freeze at −25°C. in numerous aliquots, and samples were withdrawn as needed. Unless otherwise indicated, the water-insoluble components of the homogenates were not removed. Lens antigens from other species and other rabbit tissues for testing purposes were prepared in the same way.

Five groups of adult female chinchilla rabbits (5 to 6 pounds) were immunized. The largest group of 12 received *adult rabbit lens* incorporated in Freund's adjuvant mixture (11). The final concentrations of the various components were:

Adult rabbit lens homogenate (100 mg. wet weight/ml.)	10 ml.
Mineral oil (bayol F, Esso)	8.5 "
Arlacel A (Atlas)	1.5 "
Mycobacterium butyricum (heat-killed)	20 mg.

The second group of 6 animals received approximately the same dosage of rabbit lens in 0.85 per cent NaCl suspension. The third group of 6 received the adjuvant mixture alone, in which the lens was omitted and replaced by saline solution. The fourth group of 4 rabbits received the same dose of rabbit lens in saline at the sites of injection of a streptolysin "O" concentrate (0.2 mg./0.1 ml.) given about ½ to 1 hour previously. This was prepared from the C203S strain of beta hemolytic streptococcus group A, according to the method previously described (26). The potency was about 12,000 hemolytic units/mg. The fifth group of 2 rabbits received only the streptolysin "O" concentrate. The schedule and doses used for immunization are noted in Table I. A total of 1 ml. of antigen was given at each injection into 6 separate sites, each of 0.1 or 0.2 ml. The sites were widely separated (each flank, upper and lower back) to insure extensive lymphatic drainage. The total amount of lens substance administered, therefore, represents approximately 350 to 400 mg. (wet weight) or about 130 mg. protein, estimated on the basis of total nitrogen analysis. The bleedings were done from the central ear arteries, and the sera stored aseptically at 4°C.

For the precipitin tests in agar, a modification of the Ouchterlony technic was used. Details of this method are recorded in a previous publication (27). Development of the bands with these systems usually appeared at 3 to 5 days, and reached a peak of clarity and separation at about 1 week. During the 2nd and 3rd weeks, the bands tended to become thicker and more diffuse, and the picture somewhat confused as overlapping became serious. The bands were best visualized against a dark background with a fluorescent light.

The hemagglutinin technic used was adapted from similar methods described for the study of other protein antigens (28). Details of the procedure follow: one hundred ml. of sheep blood was added aseptically to 120 ml. of autoclaved Alsever solution. This blood can be used for at least 4 weeks if stored in the refrigerator. The Alsever solution contained: glucose, 2.05 gm.; sodium citrate-$2H_2O$, 0.8 gm.; NaCl 0.42 gm.; and 100 ml. of H_2O. The pH was adjusted to 6.1 with 10 per cent citric acid.

TABLE I

Schedule of Lens Immunizations

Injection dates	Bleeding dates	Dose of rabbit lens, *mg. wet weight*				
		I Adjuvant cont.	II Rabbit lens in adjuvant	III Rabbit lens in saline	IV Streptolysin "O" plus rabbit lens	V Streptolysin "O" alone
Mar. 8, 1954	Feb. 11, 1954	0	50*	100	50	0
Mar. 16	—	0	50	25	50	0
Mar. 23	Mar. 29, Apr. 8, Apr. 19	X	X	25	50	0
May 7	May 17, June 1	0	50	50	50	0
June 9	June 17, July 14	0	50	50	50	0
July 22	July 30, Aug. 25	0	50	50	50	0
Oct. 21	Oct. 28	0	50	50	50	0
Mar. 7, 1955	Mar. 29	0‡	50	50	X	X
Total dosage of lens.....		0	350	400	350	0

X not injected.

* Dose recorded is the wet weight of lens, about ⅓ of this value is protein. The volume given at each dose was 1 ml. divided into 6 sites intracutaneously.

‡ Not all the rabbits received this dose, only 6, 7, and 5 of groups I, II, and III respectively.

For the tanning procedure, the amount of blood needed was centrifuged and the sedimented cells washed 3 times with a mixture of equal parts of 0.158 M NaCl and 0.158 M sodium phosphates at pH 6.7. The red cells were then suspended to a 25 per cent concentration (by volume) in the saline phosphate solution. To this suspension was added an equal volume of tannic acid solution (Mallinckrodt, 1:20,000 in 0.158 M NaCl). This was incubated for 10 minutes at 37°C., then centrifuged and washed 4 times with saline phosphate. After the final washing, the tanned cells were suspended to the same volume as the suspension prior to tanning. The diluent used here was 0.1 per cent bovine serum albumin (Armour, Fraction V) dissolved in the same saline-phosphate solution as above at pH 6.7. This 25 per cent tanned red cell suspension (TA-RBC) was stored in the refrigerator overnight.

To four volumes of saline phosphate, was added one volume of adult rabbit lens homogenate at 100 mg. wet weight/ml. in saline (clarified by centrifugation at 7,000 R.P.M.). One

volume of tanned red cell suspension was then added with thorough stirring. The mixture was allowed to stand at room temperature for 10 minutes, then centrifuged. The lens-adsorbed red cells were washed with 0.1 per cent bovine serum albumin (BSA) solution four times at the centrifuge to remove all non-adsorbed lens antigen. The suspension was finally made up to the volume of tanned red cells used in this last step. This 25 per cent lens-coated tanned red cell suspension could be stored for use in the ice box for at least 2 days. In the actual test, this erythrocyte suspension was diluted 1:20 with 0.1 per cent BSA to a final concentration of 1.25 per cent.

For the assay of the anti-lens sera, twofold dilutions were made by the dropping volume technic, the diluent being 0.1 per cent BSA, and the final volume being 0.8 ml. To the dilutions, 0.2 ml. of the 1.25 per cent suspension of lens-adsorbed tanned red cells was added and thoroughly shaken. After incubation in a water bath at 37°C. for 30 minutes, the tubes were shaken again and placed in the ice box overnight. The assays were read in the morning according to the pattern of the sedimented cells (see reference 24). The results were clear cut and unequivocal. The titers were recorded as the highest dilution showing definite evidence of agglutination.

In the few instances in which horizontal paper electrophoresis was carried out, the open strip technic of Grassmann was used (29), at pH 8.6 in a veronal acetate buffer, ionic strength 0.1.

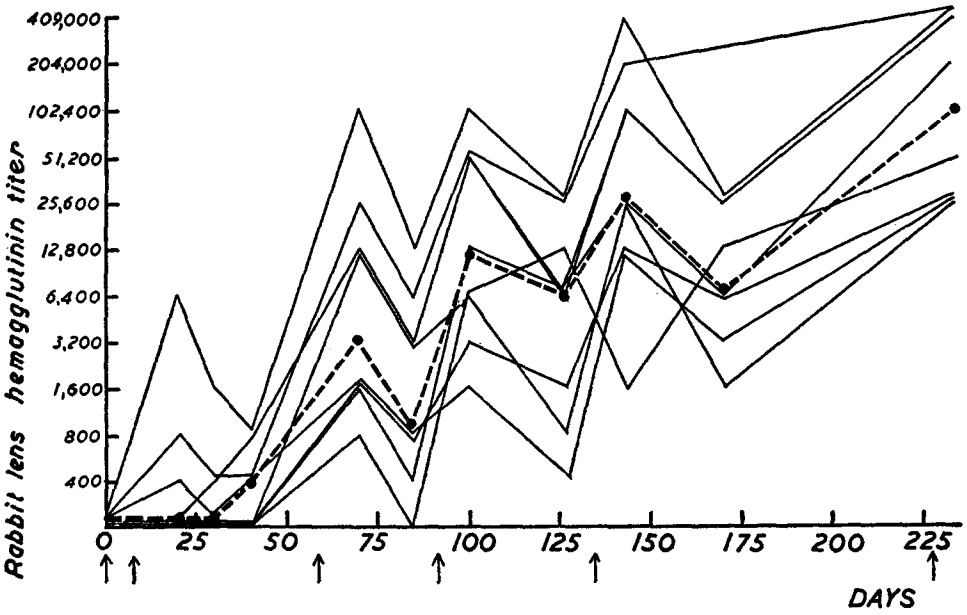
For the chemical fractionation of rabbit lens, the method of Krause (30) was used, but none of the fractions were dried. The solutions were stored frozen at −25°C.

RESULTS

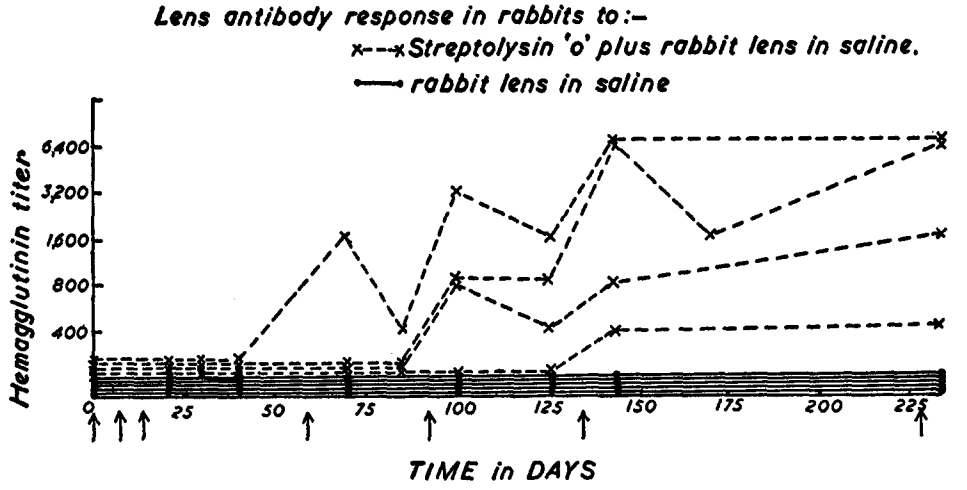
All 12 of the rabbits receiving the adult rabbit lens incorporated in Freund's adjuvants developed high levels of circulating antibody. These could be detected by the hemagglutination and agar precipitin technics, as well as by capillary precipitin tests. In Text-fig. 1 is shown the lens hemagglutinin response in the 9 animals which were followed over the entire course of the immunization procedure. The variability of the response of different rabbits may be noted, as well as the extremely high titers eventually obtained in some (1:409,000). It may be stressed that these figures represent *serum* dilutions. It may also be seen that the antibody response tended to reach a peak within 1 week or so after the injections, then to fall somewhat by 3 to 4 weeks. This pattern is somewhat at a variance with the slow attainment of peak titer, with prolonged maintenance of high antibody levels which this adjuvant mixture usually induces (31).

In those instances in which both the hemagglutinin assays and capillary precipitin titrations were done, only a very rough correlation was observed. Many samples had hemagglutinin titers from 400 to 12,800 without revealing detectable precipitins by the capillary tube method. On the other hand, all but 1 of 18 which showed capillary precipitins in *antigen* dilutions of 1:10 or greater had hemagglutinin titers of 1:1,600 or higher. All of 9 sera showing capillary precipitates in antigen dilutions of 1:90 or greater had hemagglutinin serum titers of 1:6,400 or more.

The four rabbits which received homologous lens and streptolysin "O" concentrate all showed lens hemagglutinin responses, although the titers were



TEXT-FIG. 1. Homologous lens antibody responses in rabbits with Freund's adjuvants. The arrows represent immunizing injections.



TEXT-FIG. 2. Homologous antibody responses in rabbits receiving streptolysin "O" concentrates followed by lens homogenate in saline. Lack of response in rabbits only receiving the saline lens homogenate is also shown. The arrows represent immunizing injections.

rather lower than those seen in the lens adjuvant group (Text-fig. 2). Only 2 of these rabbits showed detectable agar precipitins at any time. In addition, the antibody response tended to develop at a later period than in the adjuvant group.

All six of the rabbits which were injected with the equivalent dose of lens homogenate in saline failed to develop detectable antibodies by any of the technics employed. The responses of the five animals which were followed during the entire course of immunization are also shown in Text-fig. 2. As to be expected, the group of 6 which received only the adjuvant mixture without lens, and the group of 2 which received only the streptolysin "O" failed to show any anti-lens antibodies at any time.

Agar Precipitin Results.—During the early course of the immunizations, usually only one precipitin band was evident when tests were carried out with adult rabbit lens homogenate. With further injections of antigens, the number increased frequently to 3 or 4, and with the most potent sera, five components were clearly visible at the peak of development of the bands. The types of results are shown in Fig. 1.

It may be noted that the multiple bands may be well separated, and may vary in the intensity of precipitate as well as in the crispness of the deposit. Precipitin bands never appeared in sera that had lens hemagglutinin titers less than 1:1,600, and there was a distinct tendency for a larger number of bands to be found with increasing hemagglutinin levels. In addition, with stronger sera some of the bands tended to be much heavier than the others which could be rather faint. Visualization of these latter components was often obscured, therefore, by the width of the heavier "major" systems as the precipitin reactions developed.

Only two rabbits of the four immunized with streptolysin "O" and rabbit lens showed agar precipitins against the rabbit lens homogenates. With later specimens, some of these sera showed two bands. As to be expected, none of the rabbits immunized with rabbit lens in saline, with adjuvants alone, or with streptolysin "O" concentrate alone, showed agar precipitin bands.

That these antibody responses were specific for the lens homogenates was shown by completely negative precipitin tests in agar with all of the sera, and pools of homogenized rabbit brain, skin, kidney, liver as well as plasma. No bands were found with the anti-lens sera and these tissues, nor was there ever any effect revealed when these preparations were placed in antigen wells adjacent to the adult rabbit lens homogenate. Had similar antigens been present even in small amounts in these tissues, the bands should have shown a tendency to be distorted at the ends nearest the well containing a similar antigen. Although none of these lens antigens were present in the other non-ocular tissues examined, tests are planned with other ocular fractions. This is specially

of interest in view of the findings cited above by Rao *et al.* (22) with *heterologous* bovine lens antisera prepared in the rabbit.

It was felt of some importance to determine the effect of dilution of the homologous lens antigen on the number and character of the bands found. For this purpose, serial twofold or threefold dilutions of the rabbit lens homogenate (100 mg. wet weight/ml.) were made in 0.85 per cent NaCl. These were tested by agar precipitins and typical results are depicted in Fig. 2, with two antisera of differing potencies.

It can be seen that the band closest to the antigen well disappears upon threefold or fourfold dilution of the lens. This, as well as its position, indicates that the antigen causing this band is present in low concentration, relative to the antibody specific for it. It may be pointed out that upon dilution, the bands definitely pursue different courses, and become more clearly separated. This finding itself furnishes almost complete proof of the distinctiveness of the substances causing these reactions. It may also be stressed that at least two of these antigens appear to be present in rather high concentrations. Thus in Fig. 2 *c*, the dilution of 1:81 of the homogenate still revealed two bands, while the dilution of 1:243 showed a trace of one. The *total* soluble protein concentrations at these dilutions are approximately 0.3 and 0.1 mg./ml., and these wells received approximately 45 μg. and 15 μg. of *total* protein. It is apparent that the antigens causing these bands must make up a large proportion of the original homogenate because these amounts begin to approach the limits of sensitivity reported for this technic. As to be expected, in agreement with others, the bands became displaced as the antigen was diluted.

In Fig. 2 *d* is shown a picture of the same plate as in 2 *b*, after 2 weeks of development. The tendency of the bands to widen and become fuzzy is readily apparent. There is a strong suggestion that another component has been unmasked at the 1:32 dilution (see arrow). Recent fractions obtained with continuous flow electrophoresis support this indication that at least one component is present whose band is often masked by overlapping during development of the others. It may be mentioned in passing that the same number of components were always found with the lens homogenate whether or not it had been thoroughly clarified by centrifugation at 12,000 R.P.M. in the No. 40 rotor of the Spinco preparative ultracentrifuge (Specialized Instrument Co., Belmont, California) for a half hour.

Preliminary tests were carried out with chemically separated rabbit lens fractions, according to the technic of Krause (30). The alpha crystalline fraction was dissolved in one-half of the original volume of the rabbit lens homogenate (100 mg. wet weight/ml.), and the beta crystalline and albumin were dissolved up to the original volume in 0.85% NaCl. These fractions were tested against a potent serum in antigen wells adjacent to the original rabbit lens homogenate. The alpha crystalline mainly showed a single heavy band in the early precipitin

development (6 days), and this band joined only the middle heavy band of the 5 seen with the original homogenate. The beta crystalline component showed two bands under the same conditions, one joining the heavy band of the whole lens homogenate closest to the serum well, and the other joining the same band as the alpha crystalline. The albumin fraction showed a weak single band with the same joining reaction as the alpha crystalline. A very few tests were carried out with fractions collected by eluting appropriate portions of a horizontal paper strip on which rabbit lens had been electrophoretically separated. Although these eluates did show bands, the procedure proved unsatisfactory, and current studies are stressing the continuous flow electrophoresis technic of Durrum (32) in attempts to purify and characterize these antigens.

A number of tests have now been carried out to determine the antigens responsible for the lens species' cross-reactivities. The rabbit anti-rabbit lens sera were tested against the homologous lens homogenate while lenses of other species were put into adjacent wells. In this way human, beef, guinea pig, mouse, rat, Java monkey, *Macacus rhesus* monkey, frog (*Rana pipiens*), fish (menhaden), and chicken lenses were tested for cross-reacting components. The type of results that were obtained is represented in Fig. 3. Although the faintest bands do not show up in the reproductions, it is quite clear that some of the lens cross-reactions were due to similarities of many, if not all of the rabbit lens components. Thus in Fig. 3 *a*, the guinea pig and rat were seen to possess five cross-reacting antigens, while the mouse only seemed to show four. The frog lens revealed three heavy bands, while a very faint fourth, not seen in the photograph, was present closer to the antigen well. It may also be noted that the components nearest to the serum well with guinea pig and rat lens seem to merge with the thickened group of bands seen with the homologous system, again indicating the multiplicity of components represented there. It is also clear that some species show very limited numbers of cross-reacting antigens. Thus, the rooster lens in Fig. 3 *d* showed only two bands of the four seen with the homologous system, and one of these was too faint to be seen photographically. The menhaden lens showed only one very faint band (not seen in the reproduction) with even the strongest sera. In addition, it may be noted in Fig. 3 *e* that the homologous rabbit system seemed to show one component not seen with the *rhesus* monkey, beef, or human (6 year) lenses. In current studies, heterologous lens antisera (*e.g.* anti-frog lens sera prepared in the rabbit) are being used to confirm and extend these data in more detail. Preliminary results, in general, agree with the number of cross-reacting components found with the homologous anti-rabbit lens sera.

Similar tests were carried out in which adult rabbit lens antisera were tested against newborn and 26 day old embryonic rabbit lens (see Fig. 3 *d*). In these tests with a number of antisera, there was a consistent absence of the faint band close to the antigen wells, although the other components seemed to be

present. Since the homogenates were made on a basis of wet weight (all 100 mg./ml.) it is only possible to state that this antigen is either absent in the newborn or 26 day fetal lens, or is present in concentrations too low to detect.

It is of a great deal of interest in this connection that newborn rabbit lens and adult rabbit lens showed the same patterns on horizontal paper electrophoresis. No change in the pattern was observed when a mixture of newborn and adult lens was analyzed by this method, and compared to the separate homogenates. The adult lens antigen which *appears* to be absent in the newborn lens thus does not apparently represent one of the three major electrophoretic components (see reference 5).

A few tests have been carried out with lenses of 10 and 19 day post-partum baby rabbits in a similar way. The findings suggested that the "adult" antigen is present in barely perceptible quantities at 10 days' post partum and is readily found at 19 days after birth.

In addition, several adult rabbit lenses were dissected into cortex and nucleus as carefully as possible, and homogenates prepared of both fractions. These were tested against adult lens antisera in the usual way. The results indicated that the antigen which seems to be absent from the fetal lens predominantly resides in the cortex of the adult.

DISCUSSION

The data presented here demonstrate clearly that adult rabbit lens homogenate *is* quite antigenic in adult rabbits when adjuvant vehicles are utilized. It is also clearly evident that the rabbit lens contains at least 5 components which can act as foreign substances in the homologous animal. Although the lens antibody responses were rather variable from animal to animal, all rabbits produced high titers of lens hemagglutinating antibodies within several months. These homologous lens antibodies did not react with any of the non-ocular rabbit tissues tested. However, the cross-reactions of the homologous anti-rabbit lens sera with the lenses of other species were found to be due to the antigenic similarity of from one to all five of the definitely observed lens components. On the basis of suggestive "reactions of partial identity" seen in the agar precipitin plates, it is quite possible that some components of rabbit lens may only be partially similar to the same components in other vertebrate lenses. Further study with absorbed sera and with heterologous antisera will be necessary to determine the closeness of the antigenic relationships.

An indication was found that newborn and 26 day rabbit fetal lenses are either lacking one of the antigens present in the adult lens, or have a low concentration of this component on a wet weight basis. It is conceivable that this apparent lack is merely due to a difference in hydration. Should the absence prove to be real, the antigen may appear *de novo* as part of the developmental

process. However, since the electrophoretic patterns of adult and newborn rabbit lens seem to be the same, it is conceivable that a component is present in the newborn lens which is destined to possess adult specificity, but which has a fetal specificity at that stage of maturation. This observation is in general agreement with the studies of Burke *et al.* (33) who presented evidence that changes in antigenicity occur in the chick embryo lens during the developmental process. Although Burke's conclusions have been questioned by Ten Cate and van Doorenmaalen (34) on technical grounds, it may be pertinent to cite well documented observations along similar lines in relation to the development of human hemoglobin. Clear cut immunologic differences between fetal and adult hemoglobin have been demonstrated (35, 36). Only very small amounts of fetal hemoglobin persist into normal adult life. Recently a third normal hemoglobin has been found which appears before fetal hemoglobin, and apparently gives way to it (37). It is possible that a similar succession of biochemical specificities may also occur in the lens. An intriguing thought is that such changes may, to a limited degree, recapitulate phylogeny in the biochemical sense. Such a working hypothesis is currently being used in an extension of the observations on lens reported here.

The isolation and identification of the lens components causing these bands have only just begun. It seems clear that the observations presented here will necessitate a reorientation of thinking with regard to the chemical composition of the lens. That components other than the three "known" alpha, beta, and gamma crystallines are present has been definitely demonstrated. In addition, it is by no means certain that these crystallines identified by older technics are indeed biochemical entities, and that any or all of them are responsible for some of the antigen-antibody systems found here. It would appear that the full characterization of lens proteins and antigens must await more intensive physical, chemical, and immunological analysis than has been heretofore applied to these materials.

The data obtained with the streptolysin "O" concentrate clearly indicate that this material can also act as an adjuvant in the formation of *homologous* lens antibody, although not as effectively as the oil emulsion of Freund. Although the results of Swift and Schultz (25) indicate that staphylococcal toxin preparations did not enable rabbits to produce antibodies against rabbit lens, it is conceivable that their negative results may have been due to the relatively insensitive testing technics used. The lens hemagglutinin test has proven to be by far the most delicate used here for the detection of anti-lens antibodies. The high degree of sensitivity to small amounts of antibody is in full agreement with those who have used this method for other antigens (24, 28). At any rate, in view of this adjuvant effect, it should be of much interest to see whether streptolysin "O" fractions are also capable of bringing about the production of

other homologous tissue antibodies, notably against kidney or heart. Such studies are being undertaken.

It need hardly be mentioned that the approaches used here may be of much value in the analysis of anti-brain and anti-testis antibodies, especially with regard to the extent and number of the species, cross-reacting antigens.

SUMMARY

Five lens antigens of rabbits can induce antibodies homologously as evidenced by agar precipitin technics.

Cross-reactions of lenses of other species (organ specificity) may be due to similarity of varying numbers of these antigens.

Newborn and 26-day embryo rabbit lenses seem to have decreased concentrations of, or lack, one antigen present in adult rabbit lens.

Streptolysin "O" concentrates appear to act as an adjuvant for homologous lens immunization in rabbits.

BIBLIOGRAPHY

1. Uhlenhuth, P. T., Zu Lehre von der Unterscheidung verschiedener Eiweissarten mit Hilfe spezifischer Sera, Festschrift zum 60 Geburstag Robert Koch, Jena, Gustav Fischer, 1903, 49.
2. Hektoen, L., The specific precipitin reaction of the normal and cataractous lens, *J. Infect. Dis.*, 1922, **31,** 72.
3. Woods, A. C., and Burky, E. L., Lens protein and its fractions. Preparation and immunological and chemical properties, *J. Am. Med. Assn.*, 1927, **89,** 102.
4. Marsh, H. C., The synergic activity of staphylococcus toxin, *Yale J. Biol. and Med.*, 1944, **17,** 359.
5. Nordmann, J., Biologie du cristallin, Paris, Masson et Cie, 1954.
6. Hektoen, L., and Schulhof, K., Further observations on lens proteins, *J. Infect. Dis.*, 1924, **34,** 433.
7. Wollman, E., Gonzalez, P., and Ducrest, P., Recherches sérologiques sur les milieux transparents de l'oeil. Propriétés spécifiques du cristallin, *Compt. rend. Soc. biol.*, 1938, **127,** 668.
8. Woods, A. C., Burky, E. L. and Woodhall, M. B., The organ specific properties and antigenic power in the homologous species of alpha crystallin, *Tr. Am. Ophth. Soc.*, 1930, **29,** 168.
9. Freund, J., Casals, J., and Hosmer, E. P., Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil, *Proc. Soc. Exp. Biol. and Med.*, 1942, **37,** 509.
10. Freund, J., and McDermott, K., Sensitization to horse serum by means of adjuvants, *Proc. Soc. Exp. Biol. and Med.*, 1942, **49,** 548.
11. Freund, J., and Bonanto, M. V., The effect of paraffin oil, lanolin-like substances and killed tubercle bacilli on immunization with diphtheria toxoid and *Bacterium typhosum*, *J. Immunol.*, 1944, **48,** 325.

12. Freund, J., Thomson, K. J., Sommer, H., Walter, A., and Pisani, T., Immunization of monkeys against malaria by means of killed parasites with adjuvants, *J. Immunol.*, 1948, **28,** 1.
13. Kopeloff, L. M., and Kopeloff, N., The production of antibrain antibodies in the monkey, *J. Immunol.*, 1944, **48,** 297.
14. Kabat, E. A., Wolf, A., and Bezer, A. E., Rapid production of acute disseminated encephalomyelitis in rhesus monkeys by injection of heterologous and homologous brain tissue with adjuvants, *J. Exp. Med.*, 1947, **85,** 117.
15. Freund, J., Lipton, M. M., and Thompson, G. E., Aspermatogenesis, anaphylaxis and cutaneous sensitization induced in the guinea pig by homologous testicular extract, *J. Exp. Med.*, 1955, **101,** 591.
16. Collins, R. C., Further experimental studies on sympathetic ophthalmia, *Am. J. Ophth.*, 1953, **36,** 150.
17. Kabat, E. A., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, 1948.
18. Oudin, J., Specific precipitation in gels and its application to immunological analysis, *Methods Med. Research*, 1952, **5,** 335.
19. Ouchterlony, O., Gel diffusion methods for immunological analysis, *Sixth Internat. Cong. Microbiol.*, 1953, **2,** 276.
20. Elek, S. D., The plate virulence test for diphtheria, *J. Clin. Path.*, 1949, **2,** 250.
21. Oakley, C. L., and Fulthorpe, A. J., Antigenic analysis by diffusion, *J. Path. and Bact.*, 1953, **65,** 49.
22. Rao, S. S., Kulkarni, M. E., Cooper, S. N., and Radhakrishan, M. R., Analysis of proteins of bovine lens, vitreous and aqueous, by electrophoresis and by Oudin's gel diffusion technique, *Brit. J. Ophth.*, 1955, **39,** 163.
23. Middlebrook, G., and Dubos, R. J., Specific serum agglutination of erythrocytes sensitized with extracts of tubercle bacilli, *J. Exp. Med.*, 1948, **88,** 521.
24. Stavisky, A. B., Micromethods for the study of proteins and antibodies, *J. Immunol.*, 1954, **72,** 360.
25. Swift, H. F., and Schultz, M. P., Studies on synergy, *J. Exp. Med.*, 1936, **63,** 703.
26. Halbert, S. P., Swick, L., and Sonn, C., The use of precipitin analysis in agar for the study of human streptococcal infections. I, *J. Exp. Med.*, 1955, **101,** 539.
27. Halbert, S. P., Swick, L., and Sonn, C., The use of precipitin analysis in agar for the study of human streptococcal infections. II, *J. Exp. Med.*, 1955, **101,** 557.
28. Boyden, S. V., The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by anti-protein sera, *J. Exp. Med.*, 1951, **93,** 107.
29. Grassmann, W., and Hannig, K., Ein quantitatives verfahren zur analyse oer serumproteine durch papierelektrophorese, *Z. physiol. Chem.*, 1952, **290,** 1.
30. Krause, A. C., Chemistry of the lens, *Arch. Ophth.*, 1932, **8,** 166.
31. Freund, J., The effect of paraffin oil and mycobacteria on antibody formation and sensitization, *Am. J. Clin. Path.*, 1951, **21,** 645.
32. Block, R. J., Durrum, E. L., and Zweig, G., A Manual of Paper Chromatography and Paper Electrophoresis, New York, Academic Press, Inc., 1955.

33. Burke, V. J., Sullivan, N. P., Petersen, H., and Weed, R., Ontogenetic change in antigenic specificity of the organs of the chick, *J. Infect. Dis.*, 1944, **74,** 225.
34. Ten Cate, G., and van Doorenmaalen, W. J., Analysis of the development of the eye-lens in chicken and frog embryoes by means of the precipitin reaction, *K. Akad. Wetensch. Amsterdam, Proc. Sect. Sc.*, 1950, **53,** 894.
35. Chernoff, A. I., Immunologic studies of hemoglobin I and II, *Blood*, 1953, **8,** 399.
36. Goodman, M., and Campbell, D., Differences in antigenic specificity of human normal adult, fetal and sickle cell anemia hemoglobin, *Blood*, 1953, **8,** 422.
37. Allison, A. C., Notation for hemoglobin types and genes controlling their synthesis, *Science*, 1955, **122,** 640.

EXPLANATION OF PLATES

PLATE 31

FIG. 1. Results of agar precipitin tests with homologous anti-adult rabbit lens antisera. The sera used were from several animals at various stages of immunization, and were tested against adult rabbit lens homogenate in a concentration of 100 mg. wet weight/ml. The sera were in the large (bottom) wells, and the times of development varied from 5 days to 2 weeks. Figs. 1 *a* to 1 *e*. × 2. Figs. 1 *f* to 1 *g*. × 1.25.

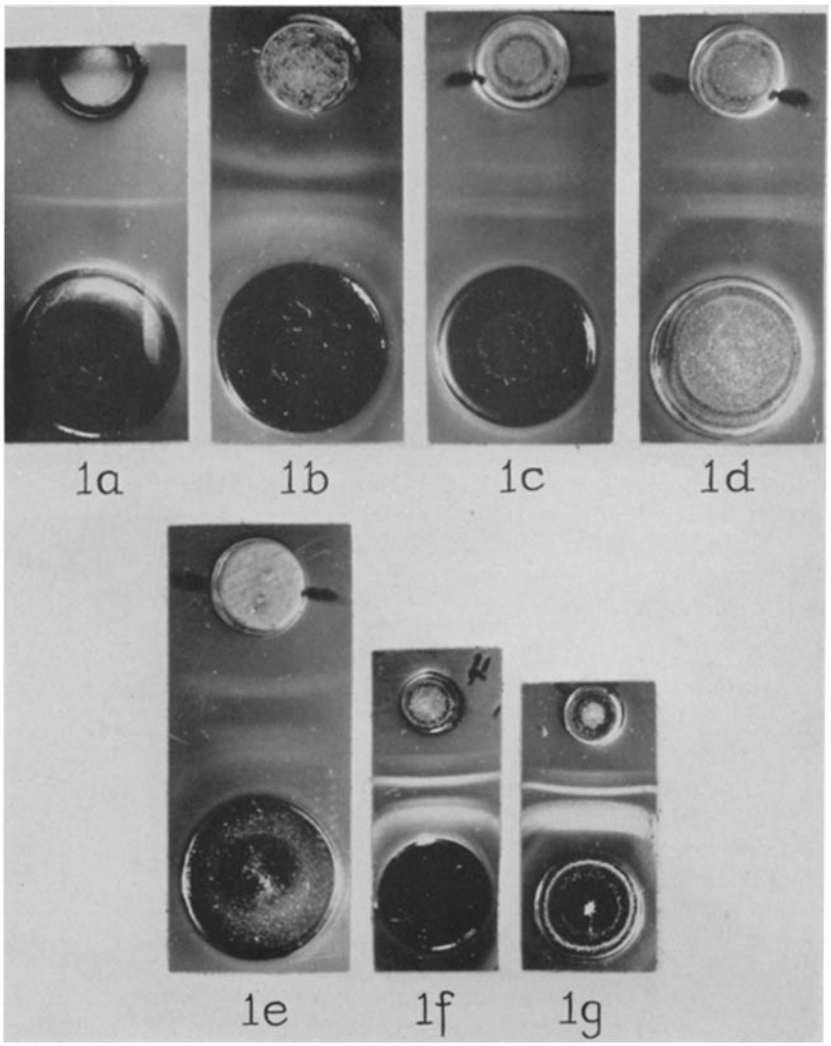


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PLATE 32

FIG. 2. Effect of dilution of homologous rabbit lens antigen on precipitin bands. × 1.

FIG. 2 *a*. Serum of moderate potency, antigen diluted serially by twofold. 6 days' development.

FIG. 2 *b*. Serum of high potency, antigen diluted by threefold. 6 days' development.

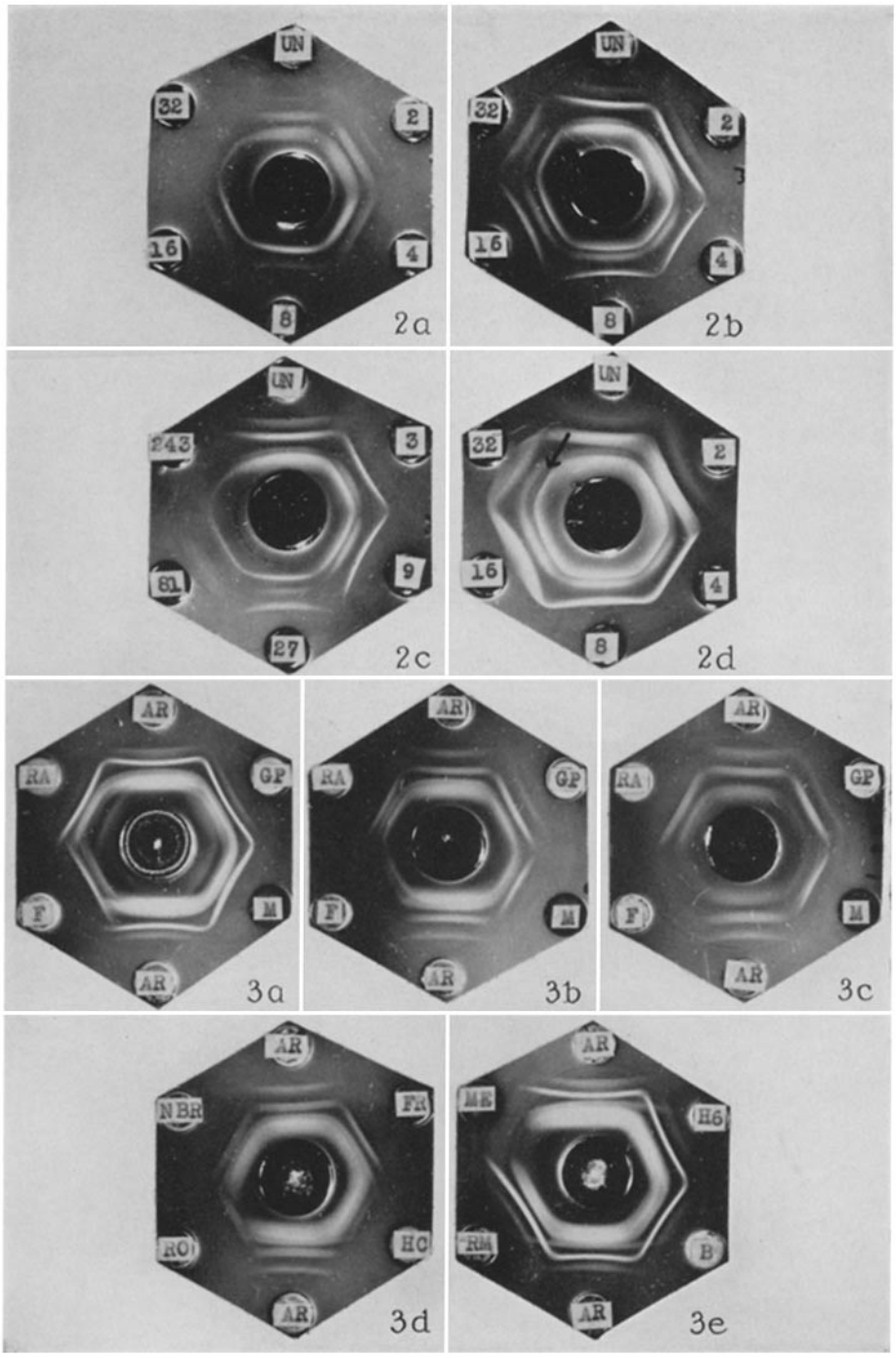
FIG. 2 *c*. Same serum as in Fig. 2 *b*, antigen diluted by threefold. 6 days' development.

FIG. 2 *d*. Same plate as in Fig. 2 *b*, after 2 weeks' development.

FIG. 3. Cross-reactions of homologous anti-adult rabbit lens antisera with lenses of other species, as well as with immature rabbit lens. × 1. Plates for Figs. 3 *a* and *e* were made with the most potent serum of rabbit 5-1, while plates for Figs. 3 *b* and *d* were made with serum of intermediate potency from the same animal from an earlier bleeding. Plate for Fig. 3 *c* was made with the most potent serum of rabbit 4-8.

Lens antigens

AR, adult rabbit	*FR*, 26 day fetal rabbit
GP, guinea pig	*HC*, human cataractous
RA, rat	*RO*, rooster
F, frog (*Rana pipiens*)	*ME*, menhaden (salt water fish)
M, mouse (albino, CF_1)	*RM*, monkey (*Macacus rhesus*)
NBR, newborn rabbit	*B*, beef
	H6, human, normal, 6 years



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