Then the groups were randomly assigned to receive one of three treatments. Both eyes of each animal were treated with the same medication. This procedure was followed to minimize the chance of administering the incorrect medication and to eliminate the influence of systemically adsorbed antiviral medication on dissimilarly treated eyes. Treatment consisted of one of the following: 3.3 per cent ARA-A ointment, 0.5 per cent IDU ointment, or petrolatum alba. A one centimeter long strip of appropriate drug was instilled into the lower conjunctival cul-de-sac every four hours for five days.

Results of treating keratitis produced by the parent McKrae strain and by the IDU-resistant herpes virus strain are reported. Fig. 1 shows the effect of ARA-A, IDU, and placebo ointments in McKrae-infected eyes after 48 hours of treatment. Severity scores for keratitis at the beginning of treatment (72 hours after infection) are not shown, but were equal in all groups. The distribution of keratitis ran from one quarter to 3+ with a median of approximately 1+. The figure shows that after 48 hours of placebo therapy, the scores for control eyes clustered at the 3+ to 4+ severity grade, while the drug-treated keratitis scores clustered at the low end of the severity spectrum. Nonparametric data analysis considering each eye as an independent variable indicates both drugs to have a highly statistically significant effect on epithelial keratitis, as compared to placebo (P = 0.001). In this experiment (and others not reported here), IDU, 0.5 per cent seems to suppress epithelial keratitis slightly more quickly and to a slightly greater extent than ARA-A, 3.3 per cent; however, the difference is not statistically significant (P = 0.10).

Results of the same therapy on keratitis produced by the IDU-resistant strain are strikingly different. In Fig. 2, note that IDU seems to have some effect as compared to placebo treatment after 48 hours, but that effect is not statistically significant (P = 0.10). By contrast, the ARA-A therapy is effective and statistically significant when compared to either IDU therapy (P = 0.01) or placebo therapy (P = 0.001).

These laboratory data demonstrate that ARA-A is effective in vitro and in vivo in treating infection produced by HSV highly biochemically resistant to IDU. These results would certainly seem to justify trial of ARA-A in unresponsive human keratitis, even if an IDU-resistant virus is suspected or encountered. However, the results of treating many cases of IDU-resistant HSV keratitis will have to be studied before ARA-A's actual efficacy in such cases in man can be determined.

Certainly, clinical and laboratory studies indicate that ARA-A may become an important adjunct to the armamentarium against herpetic keratitis, however, it has not yet been clearly demonstrated that ARA-A is actually better than IDU for general use in clinical practice. One note of caution should also be mentioned. Too few patients have been treated to know how often cases clinically resistant to ARA-A will be encountered. Such cases, including ones caused by virus strains biochemically resistant to ARA-A should be expected. Not surprisingly, we find in vitro that it is almost as easy to select for ARA-A-resistant strains of HSV (unpublished data) as it is to select for the IDU-resistant virus used in this study.

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REFERENCES

Soluble antigens of the bovine cornea. J. M. HALL, G. SMOLIN, AND F. M. WILSON, II.

The antigenic proteins in soluble extracts of bovine cornea and corneal epithelium were studied by immunoelectrophoresis and by immunodiffusion. The extract prepared from whole cornea contained alpha-, beta-, and gamma-globulins, and an albumin-like protein. The epithelium contained only traces of gamma-globulin and an albumin-like protein. A protein that appeared to be intrinsically corneal was present in both the whole cornea extract and in the epithelial extract. Soluble corneal proteins have been the subject...
of many investigations.1-4 Among proteins found by a variety of methods have been serum proteins,1-5 including immune globulins and albumin. Proteins that appeared to be present only in the cornea have been described.6 Others have investigated what they termed corneal "transplantation" antigens.6

The purpose of the present investigations was to determine the number and nature of the antigenic proteins present in an extract prepared from bovine corneas. The nature of other, nonantigenic proteins undoubtedly present in our extract was not investigated in this study.

Materials and methods. Preparation of whole corneal antigen (CA). Corneas were excised from 40 bovine eyes, frozen to facilitate cutting, and then cut into small pieces. The pieces were ground in a mortar containing phosphate-buffered saline (0.15 M, pH 7.2) and sterile sand. The aqueous suspension was incubated for four hours at 37° C., refrigerated overnight, and centrifuged at 4° C., for 40 minutes at approximately 25,000 x g. The supernatant was decanted and saved, and the pellet was resuspended in buffered saline. The centrifugation process was then repeated. The two supernatants were pooled, and the material was concentrated approximately 30-fold, sterilized by filtration through a 0.45 micron Millipore filter, and frozen in aliquots of 0.5 ml. The final total protein concentration as determined by the Biuret method was about 10 mg. per milliliter.

Preparation of corneal epithelial antigen (EPI). Epithelium was scraped from the corneas and placed in phosphate-buffered saline. The aqueous suspension of the corneal scraping was incubated for four hours at 37° C. and then processed as described above.

Preparation of antiserum. Antiserum to CA (anti-Ca), to EPI (anti-EPI), and to several bovine serum (BS) proteins was prepared in rabbits. The rabbits were given an initial intravitreal injection of 0.05 ml. of the antigen, and a subcutaneous injection of 0.5 ml. of the same material emulsified in an equal volume of Freund's complete adjuvant. The opposite eye was injected one week later. The rabbits were also injected intravenously at intervals to maintain adequate serum antibody titers. Two rabbits were immunized with EPI, three with CA, and one with each of the BS proteins.

Bovine gamma-globulin (BGG, 98 per cent Fraction II), alpha-globulin (36 per cent Fraction V, 31 per cent Fraction IV, 26 per cent Fraction III, and 7 per cent Fraction II), and beta-globulins (84 per cent Fraction III, 8 per cent Fraction IV, and 7 per cent Fraction II) were obtained from Miles Laboratories, Kankakee, Ill. Transferrin (TR) obtained from the same source, was 88 per cent transferrin. BSA (Mann Research, Burlington, Mass.) contained some BGG. The proteins were dissolved in saline at a concentration of 10 mg. per milliliter and sterilized by filtration (0.45 micron Millipore filters). Anti-BSA and antiserum to bovine serum (anti-BS) were also obtained from a commercial source (Miles Laboratories, Kankakee, Ill.).

Immunologic tests. Two weeks after their first immunizing injection some rabbits were given an intradermal injection of 0.1 ml. CA, EPI, BGG, BS, or BSA (serum protein dose was 1 mg.). The skin tests were read at 4, 24, and 48 hours.

Immunodiffusion tests were carried out on microscope slides coated with agarose dissolved in phosphate-buffered saline (0.15 M, pH 7.2, final agarose concentration 1 per cent). Immunoelectrophoresis, also performed on microscope slides, was carried out in 0.1 M barbital buffer, (pH 8.6, 250 V., 8 to 10 mA., 1 hour). The various antigens were subjected to electrophoresis, and the antisera were later placed in the troughs.

Hemolytic antibody was determined by a method described previously.7 The ability of antiserum to lyse sheep erythrocytes coated with BGG and BSA was determined.

Results. Comparison of whole CA and BS. When CA was tested against its homologous
serum in immunoelectrophoresis tests, at least six proteins were demonstrable. The results of tests on four batches of CA were essentially the same. The pattern obtained with CA and anti-CA was similar to, but not identical to, that obtained with BS and anti-BS (Fig. 1). Similarly, the pattern obtained with CA and anti-BS was not exactly the same as that obtained with BS and anti-BS. The position of several of the arcs in the CA preparations coincided with those in BS, suggesting that at least some of the proteins were serum proteins.

The position of one band in CA indicated that it was gamma-globulin. Antiserum to BGG precipitated it, and anti-CA precipitated BGG. Immunodiffusion tests showed a line of identity between anti-BGG, BGG, and CA. Although the relative impurity of the commercial alpha- and beta-globulins complicated our interpretation of test results, we were able to show that CA also contained these proteins. The rabbits immunized with CA showed positive skin test reactions to BGG and to BS and CA. The test sites were positive at four hours and remained positive for several days. The hemolytic titer of anti-CA against BGG-coated sheep erythrocytes was greater than 1:256.

One antigen in the CA appeared by its position in the immunoelectrophoresis pattern to be serum albumin (Fig. 1 and Fig. 3). Anti-BS and anti-BSA reacted with this protein by forming a precipitin arc in the position corresponding to albumin. Parallel tests using BSA in one hole and CA in the other hole confirmed that the protein migrated as albumin. There appeared to be a line of identity in the Immunodiffusion tests done with anti-BSA, BSA, and CA. However, none of our anti-CA precipitated either BSA or the albumin moiety of whole BS (Fig. 2). (The BSA preparation used did contain some BGG which was precipitated by anti-CA but, as the figure indicates, there is no arc corresponding to BSA in the pattern at the top of the figure.) Although anti-CA precipitated a protein that appeared to be BSA, the shape of the curve differed slightly from that of the anti-BSA-BSA curve. Anti-CA did not lyse sheep erythrocytes coated with BSA, and skin tests were negative.

A protein which appeared to be TR on the basis of its position in the electrophoresis pattern of CA was the major component of the EPI antigen. The precipitin line appeared very rapidly in both immunoelectrophoresis and immunodiffusion studies. Fig. 3 shows the immunoelectrophoresis of EPI and indicates that the only other components of EPI were traces of BGG and the albumin-like protein. Anti-TR did not precipitate the "strong" protein and anti-EPI did not precipitate TR. The electrophoretic mobility of the two proteins was definitely not identical. This material, which was probably not transferrin, was apparently not present in serum (Fig. 1) since anti-CA and anti-EPI did not precipitate it from normal serum, and it did not appear in the normal serum electrophoresis pattern.

Discussion. Our previous investigations indicated that cells from rabbits immunized intracorneally with BGG produced antibody specific for BGG. Preliminary experiments indicated that recipients of corneal antigen or recipients of bovine heterografts also produced anti-BGG. The present investigations were undertaken to elucidate the antigenic nature of the corneal antigen used in previous experiments. Such knowledge was felt important in order to determine the nature of the antibodies produced after immunization with the antigen or after corneal grafts of bovine cornea.

The results of our studies agree essentially with those of Watanabe and Tsutsui, Kawerau and...
Fig. 3. Immunoelectrophoresis of EPI and CA: (1) CA, (2) anti-CA, (3) EPI, and (4) nonserum protein.

Ott,2 and Holt and Kinoshita.3 Like those investigators, we were able to show that bovine corneas contained serum proteins, among which were BGG and alpha-, beta-, and gamma-globulins. Our results also agreed with previously published results in that essentially no serum proteins were found in corneal epithelium. The traces of serum proteins we noted could well have come from the stroma during the scraping process.

Holt and Kinoshita3 described a protein that comprised about 40 per cent of the soluble proteins in the epithelium and that was also one of the two antigenic, nonserum proteins in their unfractionated corneal extract. We can hypothesize that the nonserum protein in our extract, whose migration pattern resembled that of TR, is the same protein reported by Holt and Kinoshita. Preliminary experiments in our system indicate that the same protein is present in an extract prepared from stroma. It is a strong antigen and is apparently present in a rather high concentration, the precipitin arc developing within two hours in the immunoelectrophoresis tests. It is not present in serum and thus appears to be intrinsically corneal. Although, we showed that it was not TR, we cannot rule out the presence of TR in our extract. However, if it is present, it does not appear to be antigenic, or it is not sufficiently concentrated to be antigenic.

Our attempts to identify albumin in our preparation led to rather confusing results. A protein with the electrophoretic mobility of albumin was precipitated by anti-CA, and by anti-BS and anti-BSA. There appeared to be a line of identity between CA and BSA in immunodiffusion studies. However, none of our antisera to CA precipitated BSA, and anti-CA did not lyse BSA-coated erythrocytes. One rabbit, after skin testing with BSA did develop anti-BSA antibodies. The reasons for these apparent contradictory results are not clear at present.

Further efforts to characterize the corneal protein and to determine the exact nature of the albumin-like protein are in progress. Efforts to determine the relative antigenicities of various moieties among the soluble antigens are also under way.

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