Starch-gel electrophoresis of the soluble lens proteins from normal and galactosemic animals

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The soluble proteins of normal calf, rabbit, and rat lenses have been studied by the method of starch-gel electrophoresis. The electrophoretic separations of lens homogenates are compared to those of alpha, beta, and gamma crystallins prepared by paper electrophoresis and by precipitation and gel filtration techniques. Rabbit lens solutions are separated into a total of 15 protein zones, rat lens homogenates into 16, and calf into 17. Galactose cataracts which have progressed to the stage of nuclear cataract give rise to altered electrophoretic patterns. The beta and gamma protein zones decrease in staining intensity, while the alpha zone becomes very diffuse and spread out. These changes appear consistent with those observed by other techniques. Rat lenses in the prevacuolar and vacuolar stages of galactose cataract exhibit minor but characteristic alterations of certain beta protein zones. Preliminary studies of possible mechanisms of these changes are reported.

In recent years many new procedures have been made available to aid in the isolation and characterization of proteins in solution. As these methods, which include electrophoretic, immunochemical, gel filtration, precipitation, and ultracentrifuge techniques, have been applied to analysis of the soluble lens proteins, it has been shown that the classical three fractions resolved by paper electrophoresis are themselves of heterogeneous composition.[1-14] Starch-gel electrophoresis has proved to be a convenient method for qualitative characterization of the serum proteins which has demonstrated many components that were not resolved by the paper technique. In this paper, starch-gel electrophoresis has been applied to the separation of the soluble lens proteins of three species. Because many protein changes occur in cataract development, aging, and other conditions, a convenient method which allows delineation of such changes would be very useful. The second portion of this paper explores the application of starch-gel electrophoresis to the study of the protein changes which occur in the development of galactose cataracts.

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This work was supported by the United States Public Health Service Grant NB 06090, the Career Development Award S-K3-17082, and the United States Atomic Energy Commission Contract AT(30-1) 1368.

Methods

Lenses from 1 pound albino rabbits, Charles River rats, and calf eyes obtained from a local abbatoir were carefully dissected, with a posterior approach. Each lens was weighed and either frozen or used immediately. Individual lenses were homogenized in 2.8 times their weight of distilled water or a suitable buffer. Because of the large buffering capacity of the starch gel, any of

several buffers in the pH range 7 to 9 was acceptable. Individual lenses were homogenized in standard glass homogenizers, except for those of the rat, which were homogenized with a small glass rod in small (0.6 ml.) plastic tubes suitable for centrifugation. The rats were young, weighing 50 grams on the average. Lens homogenates were spun at 15,000 × g for 20 minutes to remove insoluble material. The protein concentration of the resulting supernatant was obtained with the optical density at 280 mμ. The lens solutions were then adjusted by adding buffer or distilled water to give a protein concentration of 130 to 150 mg. per milliliter. For electrophoresis with the technique to be described this concentration gives the best over-all resolution.

Galactose cataracts were induced in rats by maintaining Charles River rats initially weighing 50 grams on a diet containing 50 per cent D-galactose, as described by Kinoshita and Merola.[15] Cataracts were classified as prevacuolar, early or late vacuolar, or mature, according to criteria described earlier.[15]

The method of starch-gel electrophoresis used was that of Smithies,[16, 17] with several procedural modifications. These consisted mainly of changes in the design of the gel tray, the method of sample application, and the method of removal of background stain. The gel tray was modified so that the two widened end portions described by Smithies were eliminated, although the other dimensions were identical. Our cover lacked a slot-forming device and was of ¼ inch Plexiglas. The cathodic end of the gel tray was constructed so that, after removal of the cover, a bar of Plexiglas 2 by 16 by 0.6 cm. could be screwed into place over the upper surface of the gel. The starch gel was prepared as described[17] in the concentration recommended by the manufacturer for most studies. The lower surface of the cover was carefully covered with Saran Wrap (Dow Chemical Company, Midland, Mich.), and the cover was carefully lowered into place over the prepared gel so as to exclude air bubble formation. After the gel had cooled to room temperature, the cover, but not the Saran Wrap, was removed, leaving a smooth gel surface covered by a thin plastic sheet. In order to obtain maximum reproducibility the gel should be used within 2 to 3 hours.

At this point the protein samples are applied by means of strips of Whatman 3 MM filter paper, 0.5 cm. wide and cut to the desired length, which have previously been soaked in the lens homogenate solution. The application slot is cut in the starch gel near the cathodic end with a thin blade. The gel depth is 0.6 cm. and the strips, 0.5 cm. high, are inserted into the application slot. Paper strips cut in this way will absorb approximately 15 μl of fluid per centimeter of length so that, for example, a strip 2.5 by 0.5 cm. soaked in a protein solution containing 135 mg. per milliliter of protein will permit application of 5.0 mg. of protein. There are alternative methods of application of samples,[3, 16, 17] but in our experience this gives the most reproducible results.

After sample application, the top of the sample slot and the free edges of the Saran Wrap are sealed with warm petroleum jelly. The plastic end pieces of the tray are removed, and the Plexiglas bar described earlier, its under surface covered with a layer of petroleum jelly, is screwed into place over the gel. This bar, by exerting a mild compressing force on the cathodic end of the gel, serves to prevent buffer from the upper electrode tank from leaking down between gel and Saran Wrap and the gel and the floor of the tray when the tray is turned vertically for the electrophoretic run. Such leakage results in distortions of the protein patterns.

Contact with the electrode tanks is as described by Smithies.[17] The electrode tank buffer is 0.30M boric acid and 0.06M NaOH, pH 8.2. The gel buffer is 0.076M Tris and 0.005M citric acid, pH 8.7. Electrophoresis is conducted in a 4° C. cold room. A current-regulated D.C. power supply is set at 25 Ma. The run, usually of 3 hours duration, is considered complete when the brown line marking the buffer discontinuity has migrated 10 cm. past the starting slot. The gel is sliced and stained as described.[17] Background stain is removed in a few hours by the use of a simple electrical destaining unit designed in our laboratory. In this device, the negatively charged dye molecule is caused to migrate out of the gel toward an anode embedded in charcoal granules which absorb the stain.

Two-dimensional electrophoresis was performed according to the method of Poulik and Smithies.[18] Paper electrophoresis was in a horizontal apparatus, with the use of Whatman 3 MM paper, 0.05M barbiturate buffer, pH 8.7, 6.0 v. per centimeter, for 16 to 18 hours. In the case of calf lens, alpha, beta, and gamma crystallin fractions were eluted from appropriately cut paper electrophoretic strips. After dialysis against water, the proteins were lyophilized and redissolved in concentrations appropriate for starch electrophoresis.

Alpha, beta, and gamma fractions from calf, rabbit, and rat lenses were prepared by the methods of Spector[12] from pooled lenses. In this procedure, beta and gamma crystallins are precipitated with zinc glycinate. After the precipitate has been removed by centrifugation, the alpha fraction is purified by isoelectric precipitation. The beta and gamma precipitate is redissolved with EDTA. Beta and gamma crystallins are then separated by gel filtration in Sephadex G-75.

Spector[12] has demonstrated that the alpha, beta, and gamma fractions so prepared correspond to those separated by paper electrophoresis.

Results

The soluble proteins of calf lenses are separated by starch-gel electrophoresis into a total of 17 zones, as may be seen in Figs. 1 and 2. Each protein zone has been classified as being of alpha, beta, or gamma origin on the basis of the starch-electrophoretic patterns produced when the alpha, beta, and gamma crystallins isolated by the techniques of Spector[12] are separated, as in Fig. 1. Confirmation of this classification scheme was obtained by the two dimensional–paper starch technique, in which the lens proteins are first separated by paper electrophoresis. Each of the three paper electrophoretic zones is then subjected to further electrophoresis in starch. In general, the paper-starch technique was less satisfactory than, but consistent with, the other technique. Approximately 50 calf lenses were analyzed and found to produce the types of pattern seen in Fig. 2.

Alpha crystallin gives rise to a single, heavily staining and somewhat diffuse zone of relatively slow mobility and a second sharply defined, more rapidly migrating zone which stains less intensely. The same type of alpha pattern was produced by material eluted from paper electrophoretic strips and by two-dimensional electrophoresis, except that the "fast alpha" zone is present in such minute amounts that it is often not discernible. It was of interest to attempt separation of the alpha fractions described by Francois and associates.[5] Calf lens alpha-1 and alpha-2 fractions prepared as described were found to contain many beta and gamma contaminants un-

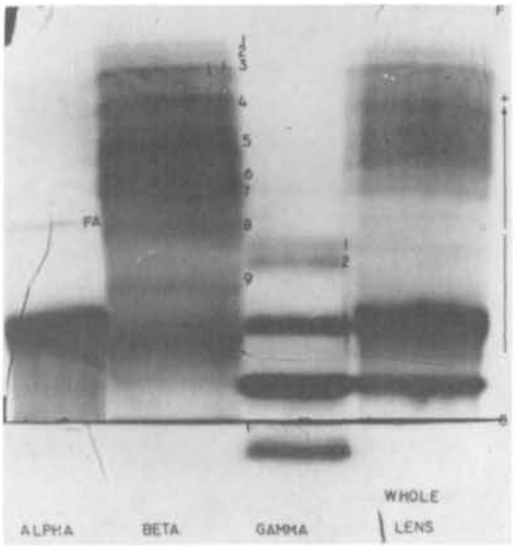

Fig. 1. Starch-gel electrophoretic patterns produced by calf alpha, beta, and gamma crystallin fractions isolated by the precipitation and gel-filtration methods of Spector.[12] Protein zones arising from each fraction are numbered in order of decreasing mobility in starch-gel electrophoresis. Protein concentrations: alpha, 40 mg. per milliliter; beta, 140 mg. per milliliter; gamma, 90 mg. per milliliter; whole lens, 140 mg. per milliliter. *FA*, "fast-alpha" zone; *F*, buffer front; *O*, sample insertion slot. The concentrations of beta and gamma fractions are relatively greater than in the whole lens homogenates in order to demonstrate the presence of faint zones, such as gamma-1 and gamma-2.

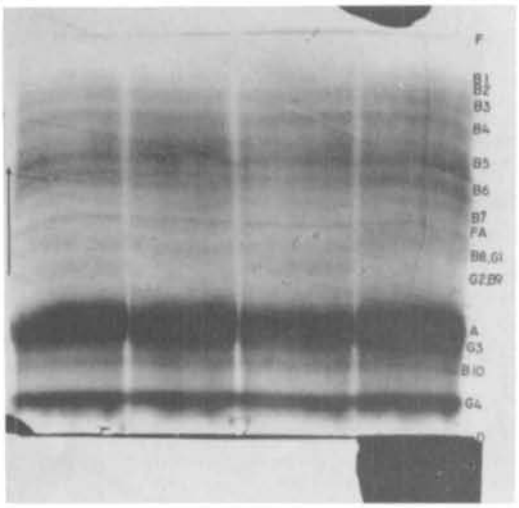

Fig. 2. Starch-gel electrophoresis of calf lens protein homogenates. The arrow indicates the direction of migration from cathode to anode. Lens homogenates from 4 different lenses run in the same gel. Protein zones are labeled according to the fraction of origin and in order of decreasing mobility: *O*, sample insertion slot; *F*, buffer front; *A*, alpha; *B*, beta; *G*, gamma; *FA*, "fast-alpha" protein. Protein concentrations, 135 mg. per milliliter.

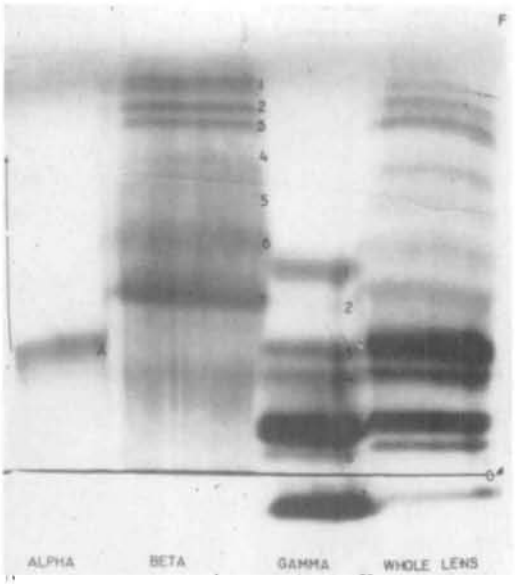

Fig. 3. Starch-gel electrophoretic separation of alpha, beta, and gamma crystallin fractions of the rabbit lens, isolated by the methods of Spector[12] and run in the same gel as a whole lens homogenate. Protein concentrations: alpha, 40 mg. per milliliter; beta, 140 mg. per milliliter; gamma, 80 mg. per milliliter; whole lens, 140 mg. per milliliter. Labels are as in Fig. 1.

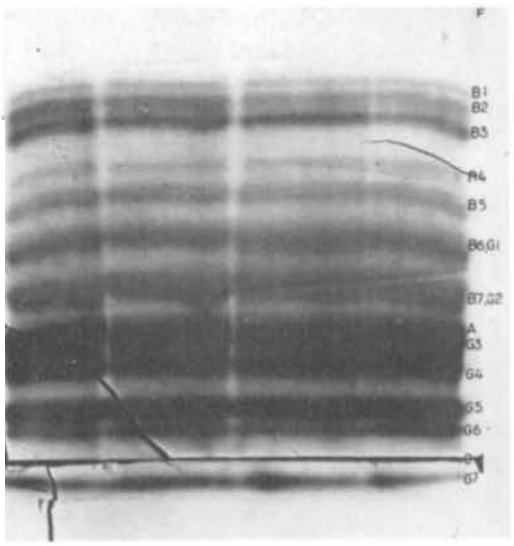

Fig. 4. Four normal rabbit lenses separated by starch gel electrophoresis. Protein concentrations, 145 mg. per milliliter. Labels as in Fig. 2.

less reprecipitated three times. The difference in starch electrophoretic mobility and staining characteristics between such purified alpha-1 and alpha-2 fractions is very slight, if present at all.

Calf lens beta crystallin prepared by Spector's method[12] is separated by starch-gel electrophoresis into a total of 10 protein zones. These zones have been numbered in Figs. 1 and 2 in order of decreasing mobility. A comparison of the isolated beta separation with that of the whole lens homogenate reveals that many zones seen in the beta preparation overlap with either alpha or gamma zones in the whole lens homogenate preparation. It should also be noted that, with the isolated fractions such as in Fig. 1, quite high concentrations of protein have been applied in order to demonstrate zones which normally are very faintly stained. A good example of this is zone gamma-1. Calf gamma crystallins are seen in Fig. 1 to separate into 5 protein zones. The two most rapidly migrating zones have approximately the same mobility as beta zones 8 and 9 and often obscure the resolution in the area of the gel when homogenates of whole lenses are analyzed. The gamma-3 component migrates with the same velocity as alpha. One gamma fraction is seen to carry a net positive charge at the pH used and hence migrates toward the cathode. Two-dimensional electrophoresis of gamma and beta proteins eluted from paper electrophoretic strips yielded results essentially similar to those in Fig. 1, although with poorer resolution.

Rabbit lens homogenates gave rise to starch-electrophoretic patterns with many similarities to, but some differences from, those of calf lenses. Fig. 3 presents the results of electrophoresis of isolated alpha, beta, and gamma fractions, while Fig. 4 shows the patterns produced by 4 individual lenses from 2 rabbits. There is in Fig. 3 a single, broad alpha zone which resembles that of calf lens. No fast-alpha zone was observed in either the isolated alpha fraction or in whole rabbit lens homogenates in spite of careful attempts to demonstrate one. Seven beta zones are

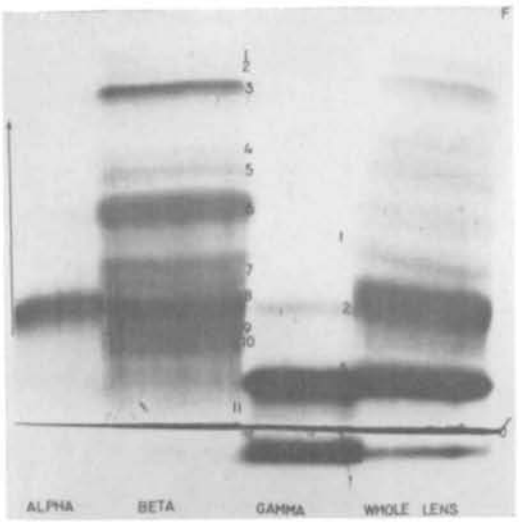

Fig. 5. Alpha, beta, and gamma crystallin fractions from 40 pooled rat lenses, prepared by the methods of Spector,[12] run in the same gel as a whole rat lens homogenate. Protein concentrations: alpha, 40 mg. per milliliter; beta, 137 mg. per milliliter; gamma, 90 mg. per milliliter; whole lens, 140 mg. per milliliter. Note that beta and gamma proteins are present in relatively greater concentrations than in whole lens homogenates.

resolved which migrate, in the main, more rapidly than either alpha or gamma crystallins. Seven gamma zones may be discerned, with the 2 most rapid ones usually indistinguishable from the slowest 2 beta zones when whole lens homogenates are separated. This represents one disadvantage of starch electrophoresis when it is used as the sole means of fractionation. As in the calf, a gamma protein band migrating toward the cathode is noted. Thus, a total of 15 soluble protein zones may be distinguished in the rabbit lens with the use of the methods described.

The rat lens gives rise to protein patterns which are in many ways similar to those of the rabbit. This may be seen in Fig. 5, which shows the separation of alpha, beta, and gamma fractions, and in Fig. 6, which illustrates the electrophoretic separations of each of the 4 lenses from 2 normal rats. Alpha crystallin again yields a single broad zone and no fast-alpha zone. Beta crystallins separate into 11 fair-

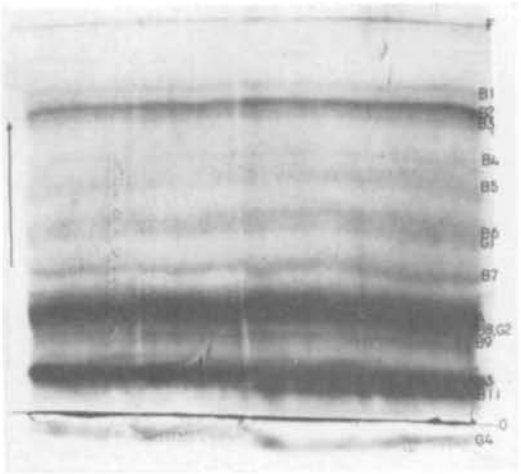

Fig. 6. Separation of normal rat lens soluble proteins. Four lenses from 2 rats. Protein concentrations are 130 mg. per milliliter. Labels as in Fig. 1.

ly distinct zones. Zones beta-4 and beta-5 are often poorly resolved. The very faint zone beta-11 is rarely seen unless high concentrations of beta protein are applied. Rat gamma crystallin produces 4 zones, one of which migrates toward the cathode. Two-dimensional paper-starch electrophoresis appears to confirm the classification given previously, although the separation of beta and gamma crystallins by this technique was not as distinct as could be wished.

Following the classification of the patterns produced by normal lens proteins in starch-gel electrophoresis, an attempt was made to determine whether the method could be used to detect protein changes occurring during the development of galactose cataracts. Within 24 hours after rats are begun on a 50 per cent galactose diet, very minor changes can be detected in the starch gel–electrophoretic patterns. The changes are noted in beta zones 6 and 7, which normally migrate somewhat more rapidly than alpha crystallin. These changes are slight, and confidence in their validity has come from their repeated presence in the patterns produced by lenses from more than 25 galactosemic rats, as well as from other results cited

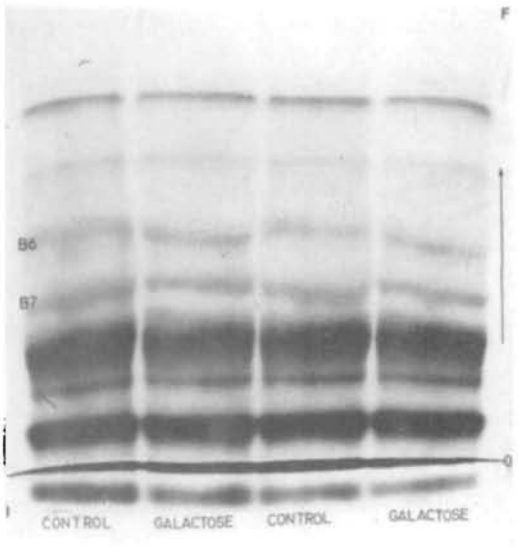

Fig. 7. Patterns produced by lens homogenates from the 2 lenses of a normal rat and a rat fed a 50 per cent galactose diet for 24 hours prior to death. Note the changes in beta zones 6 and 7 induced by galactose feeding relative to the control patterns. Protein concentrations, 140 mg. per milliliter. (See text for discussion.)

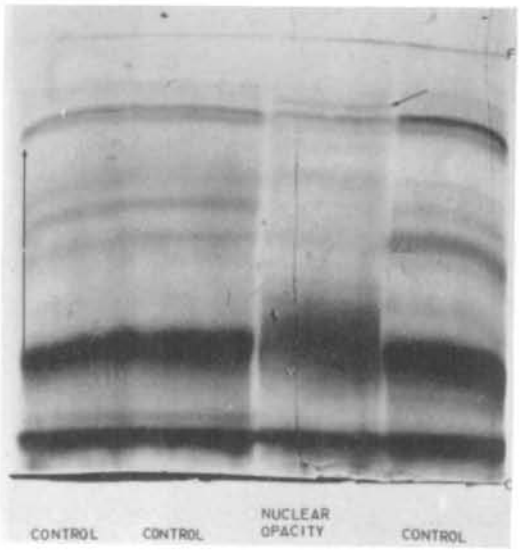

Fig. 8. Electrophoretic pattern produced by a lens bearing a dense nuclear opacity. Note the decreased staining intensity of most beta and gamma zones, the broadening of the alpha zone, and the apparently new beta zone (arrow). Protein concentrations, 110 mg. per milliliter.

later. As is suggested by Fig. 7, both zones become slightly darker staining and show minor changes in mobility, with beta-6 migrating more slowly and beta-7 slightly more rapidly. These alterations might represent a slight change in either the net charge or structure of the proteins. Preliminary results with isolated rabbit lenses incubated in a galactose medium in vitro suggest that similar changes occur in the electrophoretic beta zones 6 and 7 of these lenses after 24 hour incubations.

These altered beta protein zone patterns revert to normal if rats maintained initially on a galactose medium for 5 days are subsequently returned to a regular chow diet. This was shown in a group of rats so treated in which one rat was put to death each day. Electrophoretic patterns were compared with those of age-matched, chow-fed controls run in the same starch gel. The altered lens protein pattern gradually returned to normal over a period of 8 days after reversion to a chow diet. At the end of this 8 day period clear vacuoles could still be discerned in the lens with the ophthalmoscope.

The changes in beta zones 6 and 7 become somewhat more definite, but are otherwise unchanged, throughout the early and late vacuolar stage of galactose cataract development. However, upon the development of the stage of nuclear opacity, profound alterations of the protein patterns are observed, as seen in Fig. 8. Three principal changes may be seen. First, the gamma and beta zones are markedly decreased in their staining intensity. Nevertheless, those gamma and beta zones present in high enough concentrations to be visualized appear to retain their usual mobilities. The second major change is that the alpha region is much broader and more diffuse than normal. Third, a faint new zone appears between beta-1 and beta-2. Two dimensional electrophoresis has demonstrated that this new zone is also a beta protein.

The presence of a "fast anodic fraction," described earlier[19, 20] as occurring

in nuclear galactose cataracts, was confirmed by us with paper electrophoresis. If, after paper electrophoresis, the strip is subjected to starch-gel electrophoresis by the two-dimensional technique, the alpha protein which retains its normal mobility on paper is seen to do so in starch gel as well. It does not appear to be altered. The "fast anodic fraction," migrating more rapidly on paper, also migrates more rapidly in starch gel and gives rise to a broad, poorly resolved zone. Its slower end is fused with the normal alpha zone. This pattern suggests that the "fast anodic fraction" may be alpha crystallin which has been altered structurally in some way during the development of the mature cataract. The change in the alpha band differs from the change seen in the beta and gamma zones. The latter appears to have lost protein but retain their normal mobilities.

Experiments were conducted to seek explanations for the changes which develop in beta zones 6 and 7 when the lens is exposed to galactose. To determine whether the presence of dulcitol alone could initiate the beta zone changes, rabbit and rat lenses were homogenized in 0.125 and 0.15M dulcitol, approximately the concentrations normally found in lenses exposed to galactose.[15] Electrophoresis of these homogenates did not reveal any detectable deviation from the patterns of paired lenses not exposed to the sugar alcohol. Similarly, lenses homogenized in 30 mM. of galactose also showed no changes.

In view of Dische, Zelmenis, and Youlis's[21] demonstration of an increased susceptibility of certain lens proteins to oxidation in early galactose cataracts, the possible role of oxidative phenomena in production of either early or mature changes was explored. Lens glutathione levels decrease markedly within 24 hours after galactose feeding is begun in rats.[22] Exposure of lens homogenates containing 2×10^{-5}M cupric ion to an oxygen atmosphere for 2 hours has been shown to result in oxidation of nearly all glutathione but very few protein sulfhydryl groups.[23] Rat lenses were therefore homogenized in Tris-citrate gel buffer containing 2×10^{-5}M cupric ion and incubated in 100 per cent O_2 atmosphere at 37° C. for various periods. The opposite lenses from the same rats were treated similarly but were incubated under N_2. After 2 and 3 hour incubations, normal lens homogenates exposed to O_2 showed some changes in the relative staining intensity of beta zones 1 to 3 and a slight over-all decrease in staining of all beta zones. A 3 hour exposure to oxygen of homogenates of lenses of rats fed galactose for 4 days produced a prominence of zone beta-3 and a general decrease in staining of all other beta and some gamma zones that was more marked than was seen in oxidized normal lens homogenates. In the lenses of galactosemic rats a spreading of the alpha zone was noted. Prolonging the incubations to 22 hours produced much more marked changes. Sufficient protein was oxidized in the vacuolar stage galactosemic lens homogenates to result in a visible precipitate. Starch-gel electrophoresis showed a major loss of beta and gamma zones in homogenates of both normal and galactosemic lenses, but this was more marked in the latter. The alpha zone became very broad, suggesting a possible degradation or alteration of this protein. Thus, a removal of reduced glutathione and exposure to mild oxidizing conditions does not seem to result in changes in beta zones 6 and 7 such as those seen in the vacuolar stage of galactose cataract. Prolonged exposure does result in alterations resembling those noted in mature cataract.

Another approach to the nature of the early beta protein changes observed in the lens of rats fed galactose was through the use of starch gels containing a sulfhydryl reducing agent, mercaptoethanol. A number of normal and galactose fed (5 days) rats were killed, and all lenses were homogenized in 0.01M mercaptoethanol. A series of starch gels containing from 0 to 0.1M mercaptoethanol was made. Elec-

trophoresis revealed that the galactosemic and normal rat lens patterns were indistinguishable in gels containing more than 0.005M mercaptoethanol, while in gels containing less than this the usual alterations in zones beta-6 and 7 were seen. These results suggest that the beta protein changes involve a susceptibility to oxidation of protein sulfhydryl groups, which is reversed or prevented by the continued presence of greater than 0.005M mercaptoethanol. It should be noted that the presence of this amount of mercaptoethanol in the starch gel results in an increased endosmotic flow. This flow, which is toward the cathode and which retards the rate of migration of proteins toward the anode, lengthens the average electrophoresis time from 3 to 4½ hours. This endosmotic effect does not appear to affect the usual relationship of protein zones to each other, and there is no reason to suppose that it alters the relative mobility of the beta zones in question.

Discussion

Starch-gel electrophoresis is a relatively simple and inexpensive technique which may accomplish a more complete separation of the soluble lens proteins than any other method alone. It does not effect complete separation of many proteins in the slow beta and fast gamma groups. It requires only a few hours to complete and permits analysis of several samples in the same gel. As illustrated in the galactose cataract study, the technique appears as a feasible method to screen for lens protein changes. Of course, it does not by itself allow quantitation or characterization of the nature of any protein changes found. Smithies[16] has pointed out the gentleness of the method and observed that no artifactual alteration of proteins by the technique has yet been demonstrated. Starch gels may be made which contain up to 8M urea, mercaptoethanol, or other agents, and the pH of the gel may be varied over a fairly wide range.[16] To a limited extent, the average pore size within the gel may be varied by changing the concentration of starch, thus allowing differential retardation of different sized molecules.[24] Methods of preparative electrophoresis with starch gel have been described,[16] and these should permit isolation and further study of proteins separated by the method.

The designation of each protein band seen in starch-gel electrophoresis as an alpha, beta, or gamma crystallin must be somewhat arbitrary, since rigid criteria for defining the three crystallins have not as yet been established. The zinc glycinate method, coupled with isoelectric precipitation and separation on Sephadex G-75, as described by Spector,[12] does at least give a precise description of how the 3 crystallin fractions were obtained. Further, Spector[12] has demonstrated that the 3 fractions so obtained exhibit the expected mobilities of alpha, beta, and gamma crystallin in paper electrophoresis.

In spite of its high charge in alkaline solutions, alpha crystallin migrates only a short distance into the starch gel. This probably reflects the high molecular weight of the native protein, usually considered to be 800,000 to 1,000,000.[8, 13] Alpha is not as sharply resolved as the several beta and gamma zones. The spreading of this zone may not necessarily be a consequence of the high protein concentration in this region of the gel, since some of the gamma zones in each species stain very intensely and yet are sharply defined, narrow bands. The broad alpha zone may be due to the structure of alpha crystallin. There is growing evidence that this protein is an aggregation of a large number of subunits which may[13] or may not[9] be identical. It is uncertain at present whether these alpha aggregates exist naturally in one or in more than one principal form.[5, 8, 9, 13] In this regard, Maisel and Goodman[3, 4] conclude that two alpha zones may be resolved in two dimensional–paper starch electrophoresis, although this is seemingly at variance with the results of the present study. Maisel and Goodman[3, 4] used a much

lower protein concentration than was used in the present study, and the lenses used may have been from older animals. Furthermore, the inadequate resolution of most beta and gamma zones evident in the photographs[3, 4] makes comparison more difficult.

Several investigators, by using primarily ultracentrifugal data, have found, in addition to the major alpha protein, a minor, smaller protein in preparations of calf[1, 8, 13] and rabbit[14] lens proteins. In the present experiments, starch-gel electrophoresis of both isolated alpha crystallin and whole lens homogenates revealed a sharply defined, rapidly migrating "fast-alpha" zone in the calf but not in the rabbit or rat. The nature of this fast-alpha protein and its relationship to the minor alpha protein found by others is unknown.

As is apparent from our own and from other studies, beta crystallin contains a large number of components in the natural state. For example, immunochemical studies reveal several antigens in both beta and gamma crystallin.[1] Several peaks may be eluted from beta fractions separated by ion-exchange column chromatography[6, 12] and by gel filtration.[10] In the present study, starch-gel electrophoresis resolved 10 beta protein zones in calf, 7 in rabbit, and 11 in rat lens preparations. The molecular weight of these proteins is generally considered to be in the order of 50,000 to 100,000,[10] and they exhibit greater mobility than gamma proteins in free electrophoresis. Thus, it is not surprising that most beta proteins migrate faster in starch gel than either alpha, which is a much larger molecule, or gamma, which is less highly charged in alkaline solution.

Calf and rabbit gamma crystallin has been judged nearly homogeneous in the ultracentrifuge,[7, 13, 14] although more than one fraction may be eluted by ion-exchange chromatography[12, 14] and by a combination of gel filtration and ion-exchange techniques.[11] In the present study 5 gamma protein zones are found in calf, 7 in rabbit, and 4 in rat lens preparations. However, some of these are apparently present in very small amounts and may only be visualized by starch-gel electrophoresis when very large amounts of isolated gamma protein are applied to the gel.

The observation that protein changes in beta zones 6 and 7 occur in the lenses of rats fed galactose for more than 24 hours indicates that this is one of the earliest reported changes occurring in experimental galactose cataract. The nature of the protein changes remains obscure. The apparent increased staining intensity may reflect a slight increase in protein concentration or may merely be caused by a narrowing of the zone, with the total amount of protein remaining constant. The slight mobility changes could result from a minor alteration of either molecular size or charge. Alternatively, they might be caused by the binding of some small molecule to the protein. The fact that the presence of mercaptoethanol in the gel in concentrations greater than 0.005M reverts the altered patterns to normal strongly suggests that the changes involve sulfhydryl groups. Perhaps the beta proteins affected are related to the easily oxidizable beta proteins described by Dische, Zelmenis, and Youlis[21] in studies on galactose cataracts. The possibility that the lack of glutathione is responsible for these changes is somewhat doubtful. The loss of glutathione under mild oxidizing conditions in normal rat lens homogenates in the present study did not lead to these protein changes. Whether the altered beta proteins themselves have a causative role in the development of further lens changes, or whether their alterations are merely a reflection of a more general but undetected lens change, is not understood.

The marked protein changes observed in nuclear galactose cataracts are consistent with those described by earlier investigators.[19, 21] Thus, beta and gamma protein concentrations decrease to such an extent that many zones can no longer be

discerned. Marked permeability changes are thought to occur in the lens at this stage, and the lens proteins are not only converted to insoluble protein but also may be lost either by leakage or proteolysis. It is suggested on the basis of the mobility characteristics seen in starch-gel electrophoresis that those beta and gamma proteins which do remain in the lens at this stage are not altered structurally, with the exception of the new "beta" zone in Fig. 8. The "fast anodic fraction" observed in paper electrophoresis of nuclear galactose cataracts[19] is seen in starch electrophoresis to possibly represent altered alpha protein. The changes seen in nuclear galactose cataract may be reproduced, to an extent, by prolonged oxidation of lens homogenates, according to our data. It is probable, however, that many factors contribute to the development of protein changes at this stage.

We are grateful to Dr. T. Kuwabara for preparing the photographs seen in this paper.

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