Lenses obtained from rats on a galactose diet for six days showed a marked reduction in their capacity to incorporate $^{35}$S-methionine into protein even though there was no reduction in the capacity of the lenses to incorporate $^3$H-thymidine and $^3$H-uridine into DNA and RNA, respectively. Therefore, lenses were incubated in media in which various putative cataractogenic factors were varied to determine the effects on $^{35}$S-methionine incorporation. Methionine incorporation paralleled the size of the AIB pool rather than the chloride ratio or concentration of potassium in the lens. The size of the amino acid pool and the concentration of lens potassium are known to be lowered by lens swelling. When the concentration of potassium in the lens is lowered, along with swelling in hypotonic medium or in medium containing galactose, or when the concentration of potassium is lowered with little swelling by substitution of choline for some of the sodium in the medium, $^{35}$S-methionine incorporation is decreased. However, this decrease in $^{35}$S-methionine incorporation was prevented in all cases without a significant increase in potassium concentration if the medium was fortified with amino acids. Increasing the level of potassium in the medium increased the concentration in the lens to normal levels. However, $^{35}$S-methionine incorporation was not increased in media containing galactose or choline. Invest Ophthalmol Vis Sci 24:106–112, 1983

Cataracts associated with diabetes or galactosemia can be prevented by treatment with insulin or by the removal of galactose from the diet. The study of sugar cataracts, therefore, is primarily of interest as a means of learning more about the cataractogenic process.

The initial steps or changes in the development of sugar cataracts have been established by showing how they arise from the primary cause and by demonstrating that the reversal of the change, without affecting other factors, will delay or prevent cataracts. Elevated levels of blood glucose or galactose are established signs of diabetes and galactosemia and each has been associated with the formation of cataracts. The lowering of the blood sugar level by partial starvation or by treatment with phlorizin or insulin will prevent cataract formation. The sugars are carried from the blood into the aqueous, and hence, to the lens where they are reduced to the corresponding sugar alcohol by aldose reductase. The prevention of polyol formation by inhibiting the action of this enzyme prevents cataract formation. Thus, hyperglycemia followed by polyol formation are established steps in the cataractogenic process. The subsequent steps that lead to the formation of nuclear cataracts remain to be defined.

The accumulation of polyols in the lens is responsible for lens swelling and this in turn is responsible for a lowering of the concentrations of amino acids and potassium in the lens. When cataracts are produced under a variety of conditions, the time required for cataract formation correlates better with swelling than it does with the content of polyols per se. Therefore, swelling arising from the accumulation of sugar alcohols is probably a specific factor in the formation of cataracts.

The sudden appearance of nuclear cataracts in rats after 14 or more days of galactosemia or 70 or more days of diabetes is associated with a loss of the semipermeable characteristics of the lens membranes. It has been suggested that this may be related to a gradual decrease in Na, K-ATPase during cataractogenesis. The inhibition of this enzyme has been implicated as a factor in the development of cataracts in the Nakano mouse. In sugar cataracts the depletion of the enzyme might be explained by the fact that there is little net synthesis of proteins while cataracts are developing. Thus, the early steps of the cataractogenic process may be linked to the appearance of nuclear cataracts through a depression of pro-
tein synthesis resulting from a decrease in the concentration of amino acids or, as recently suggested, a decrease in the concentration of lens potassium. It is the purpose of this study to determine the effects of amino acids and potassium on protein synthesis, as measured by $^{35}$S-methionine incorporation, as a means of assessing the relative role of each in the formation of sugar cataracts.

**Materials and Methods**

Lenses were obtained from male Sprague-Dawley rats weighing 100 ± 20 g. The enucleated eye was washed in normal saline containing 1% of Wescodyne for at least 1 min; the excess solution was removed on a sterile towel, and the lens was dissected free, using a posterior approach, while the eye was immersed in the medium required for the experiment.

Prior to killing the rats, they were maintained on a regular diet of Purina dog chow pellets. In some instances a cataractogenic diet, consisting of a mixture of 50% ground Purina dog chow plus 50% galactose, was fed to the rats for a period before the lenses were removed.

Incubation was carried out in CO$_2$/air humidified incubators at 37 C and pH 7.4. A modified TC 199 medium was used with the final concentration (mM) of salts being 1.4 CaCl$_2$, 1.0 MgSO$_4$, 0.4 NaH$_2$PO$_4$, 0.5 Na$_2$HPO$_4$, 30.0 NaHCO$_3$, 4.0 KCl, and approximately 110.0 NaCl. The concentration of the latter was adjusted to produce a medium with an osmolality equal to 305 mOsM. The medium contained 6 mM glucose, 2.5% fetal calf serum, and the organic constituents of TC 199 (Gibco). This represents the composition of the basic isotonic medium. Changes were made as indicated in individual instances by altering the concentrations of NaCl and KCl and by the addition of other substances.

DNA and RNA assays were made after separating the total nucleic acids from lens homogenates. Lenses were homogenized with a Polytron (Tekman) homogenizer in a SET solution (347 mM sodium laurylsulfate [SDS], 50 mM Na$_4$EDTA and 100 mM Tris HCl at pH 7.5) containing 0.1 mg per milliliter of proteinase K, which is derived from a fungus (Merck). One milliliter of SET solution was used for each lens in a pooled sample. After incubating the homogenate containing proteinase K for 15 min at 37 C, any remaining proteins and lipids were removed by extraction with a half volume amount of phenol plus a half volume amount of chloroform. Following centrifugation at 8000 rpm for 15 min to separate fully the phases, the aqueous phase was mixed with two times its volume of ice cold ethanol. After storage overnight at -20 C the nucleic acids were precipitated and removed by centrifugation at 8000 rpm for 10 min. After washing with ethanol and recentrifugation the precipitate was lyophilized.

DNA was determined by measuring the fluorescence produced by the reaction of 3,5-dianinobenzoic acid with the deoxyribose moiety of DNA.

RNA was separated using a 4–20% sucrose gradient in which the sucrose is dissolved in SET solution. The nucleic acid preparation was dissolved in SET, layered over the sucrose, and centrifuged for 5 hr at 39,000 rpm at 25 C (SW 41 rotor, Beckman). Approximately 0.5 ml aliquots were collected by hand and the absorbance was measured at 260 nm. Yeast ribosomal RNA was used as a marker and run in parallel to determine the aliquots containing 18S and 28S ribosomal RNA.

The rate of DNA and RNA synthesis was measured by determining the amount of $^3$H-thymidine or $^3$H-uridine, respectively, that was incorporated in DNA or RNA after incubation of lenses for 6 hr in a medium containing the isotopically labeled nucleoside. The incubation media had a volume of 1 ml and contained 0.2 m Ci per ml with a specific activity of 50 Ci/mM.

The rate of protein synthesis was determined by measuring the amount of $^{35}$S-methionine incorporated in lenses after 6 hrs of incubation in 1 ml of medium with 3–4 × 10$^7$ cpms of $^{35}$S-methionine. The concentration of methionine was kept constant for all incubations. When media were fortified with amino acids, additional methionine was not added. After the incubation was complete, the lenses were rinsed in a “cold” medium for 10 sec and then homogenized in 1 ml of 50 mM Tris buffer (pH 7.0) containing 0.1 mM EDTA and 1.0 mM dithiothreitol (DTT). After centrifuging at 2500 rpm for 30 min at 4 C, 10 µl aliquots of the supernatant were added to equal volumes of a solution containing 2% sodium laurylsulfate (SDS), 2% dithiothreitol, 8 M urea and 10% glycerol in a 2% Tris HCl buffer at pH 7.4. The samples were then used for SDS polyacrylamide slab gel electrophoresis (SDS-PAGE). Discontinuous polyacrylamide gel electrophoresis was performed by the method of Maizel. The separating gel was a 12% polyacrylamide gel slab containing 0.1% SDS and 0.19 M Tris–HCl at pH 8.5; a 4% polyacrylamide stacking gel containing 0.1% SDS and 0.06 M Tris HCl, pH 6.8, overlaid the separating gel. Electrophoresis was for 2–4 hr at 100 V at room temperature. Following electrophoresis the gels were stained with Coomassie blue, destained, mounted and dried on filter paper, photographed, and autoradiographed. Parallel electrophoretic runs were made with proteins with known molecular weights—phosphorylase B (94 K), bovine serum albumin (67 K), ovalbumin (43 K),...
carbonic anhydrase (30 K), soybean trypsin inhibitor (20 K) and α-lactalbumin (14.4 K). Densitometric scans of the radioautographs were made with a Transidyne Scanning Densitometer. All samples that were compared contained the same amount of supernatant from the homogenate of a single lens and were incubated and subjected to SDS-PAGE simultaneously. Scans were made on the same autoradiograph with identical settings of the scanner. Conditions were not identical for different sets of autoradiographs. As an alternative method of assessing protein synthesis, a lens, following incubation in 35S-methionine, was homogenized in 1 ml of 50 mM Tris buffer (pH 7.0) containing 0.1 mM EDTA and 1.0 mM of DTT. The total lens proteins were precipitated by adding an equal volume of 10% TCA and storing at 4 C for 24–48 hrs. The precipitate was separated by centrifuging at 2500 rpm for 30 min at 4 C. After resuspending the precipitate in 5% TCA, the centrifugation was repeated, and the precipitate was dissolved in 0.5 ml of 30% hydrogen peroxide over a period of 48 hrs at 60 C. Aliquots of 10 μl were mixed with 6 ml of aqueous scintillant (Amer-sham) for counting (Nuclear-Chicago). Samples of incubation media were prepared in a similar manner for counting. The degree of 35S-methionine incorporated in the lens as determined by TCA precipitates provided a method for selecting representative samples for making SDS-PAGE autoradiographs as described earlier. The latter indicates changes in the level of soluble proteins.

Estimates of the size of the amino acid pool were made by measuring the distribution of 14C-AIB after 24 hrs of incubation in 5 cc of medium. Incubation media contained approximately 106 cpm/ml. Following incubation, lenses and 100 μl aliquots of medium were added to 1 ml of Hyamine hydroxide and scintillant fluid and counted.

The steady state distribution of chloride between lens and medium was determined as described for AIB except that 36Cl was used as the active isotope. The chloride ratio was calculated by dividing the counts per μl of medium by the counts per μl of lens water with appropriate corrections for extracellular space.

Lenses were measured after incubation. Dry weights were determined after drying to constant weight in a 96 C oven (36 hrs or more). The dry weights were in the 8–10 mg range, and all results were adjusted to a 10 mg dry weight to permit easy comparison of results. The difference between the wet and dry weights was taken as the weight of total lens water. A correction for extracellular water equal to 6% of lens water was made. Earlier studies with 3H inulin on lenses incubated for 24 hrs indicate that this is appropriate for the conditions reported in this study.26 Substances are assumed to be present in the extracellular space in the same concentration that exists in the medium. The content of a substance in the intracellular water of the lens, therefore, is equal to the content in the total lens minus the content in the extracellular space. The latter is equal to the concentration in the medium times a volume equal to 6% of the total lens water. The concentration of a substance within the lens is then equal to the content in the intracellular space divided by a volume equal to 94% of the total lens water.

Sodium and potassium levels were determined on 5 ml trichloroacetic acid extracts of the dried lenses against a series of known sodium and potassium standards using a Perkin-Elmer atomic absorption spectrophotometer.

Results

Status of Protein Synthesis in Lenses from Galactose-fed Rats

The relative degree of 35S-methionine, 3H-thymidine, and 3H-uridine incorporated in lenses from control and galactose-fed rats was determined to assess the rate of protein, DNA and RNA synthesis during galactosemia. The results as determined on six or more pooled lenses for each determination are shown in Table 1. After six days of galactose feeding, protein synthesis, as indicated by 35S-methionine incorporation, is reduced and equals about 20% of the control level. DNA synthesis, as indicated by 3H-thymidine incorporation, and ribosomal RNA synthesis, as indicated by 3H-uridine incorporation, are not reduced. In the latter instance ribosomal RNA synthesis is increased. These results are consistent with recent studies of others21 that show that the level of messenger RNA is not reduced in lenses from rats on a similar diet. The conclusion is drawn that the deficiency of

| Table 1. Effect of 6 days of galactose diet on 35S-methionine, 3H-thymidine and 3H-uridine incorporation in rat lenses* |
|-----------------|--------------|
| Methionine      | Control      | Galactose-fed |
| cpm/lens        | 48.5         | 9.0           |
| cpm/μl-Med.     | 32,299       | 27,087        |
| Thymidine       | 516,000      | 148,000       |
| (cpm/μg DNA)    | 146,000      | 516,000       |
| Uridine         | 88,000       | 148,000       |
| (cpm/A260 unit of RNA) | 24-S-RNA | 516,000 |
| 18-S-RNA        | 146,000      | 516,000       |

* Fresh lenses were incubated for 6 hrs in the basic medium and under the conditions described under Methods.
protein synthesis is related to a failure of translation. 

$^{35}$S-methionine incorporation was also determined on lenses from rats after 12 days of galactose feeding. The results indicate that the capacity of the rat lens to synthesize protein was reduced further to about 4% of the control level.

Studies with Selected Media

A TC 199 medium was modified by changing the relative concentrations of sodium, potassium and sucrose in a manner that would alter the ionic composition within the lens (Fig. 1). Four of the media (A–D) were formulated so that the volume of lens water would be constant and the fifth (E) was made hypotonic by omitting sucrose so that the lens water would be increased. Following incubation for 24 hours, the desired results were obtained. The lens water was constant except for a significant increase ($P < 0.01$) in medium E. The concentration of potassium in the lens water ($[K]_j$) was significantly increased ($P < 0.01$ compared with lenses in Medium A) where the concentration of potassium in the medium ($[K]_o$) was increased, and there was a concomitant decrease in the concentration of sodium in the lens water ($[Na]_j$). When the concentration of sodium in the medium ($[Na]_o$) was decreased without a change in $[K]_o$ (medium C vs A) $[K]_j$ was significantly lower ($P < 0.02$).

The effects of changes in the external and internal cation concentrations on the amino acid pool, as estimated by allowing $\alpha$-amino-isobutyric acid (AIB) to distribute itself between lens water and medium during 24 hrs of incubation; on the chloride concentration inside ($[Cl]_j$) and outside ($[Cl]_o$) the lens, as estimated by the distribution of $^{36}$Cl after 24 hrs of incubation; and on protein synthesis, as estimated by the incorporation of $^{35}$S-methionine in a TCA precipitate from lenses incubated with the isotope labeled amino acid during the last 6 hrs of a 24-hr incubation in a specific medium are shown in Figure 2. The results indicate that the pool of amino acids is significantly increased ($P < 0.02$) when the $[K]_o$ is raised (medium B vs A). However, this effect is not evident when sucrose is present in both media (medium D vs C). With lens swelling (medium E), the pool of amino acids is smaller. This is consistent with earlier

<table>
<thead>
<tr>
<th>Medium (mM)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[Na]_o$</td>
<td>142</td>
<td>142</td>
<td>98</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>$[K]_o$</td>
<td>4</td>
<td>48</td>
<td>4</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
<td>88</td>
<td>88</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Water content and concentrations of potassium and sodium in lenses incubated for 24 hrs in selected media. Medium A is the control TC-199 medium described in the text. Media B-E differ in their concentrations of sodium, potassium and sucrose as shown at the top (six or more lenses per medium, mean ± SE); $^*P < 0.01$; $^**P < 0.02$.

Fig. 2. Amino acid pool sizes and chloride ratios after 24 hrs of incubation of lenses in selected TC-199 media with sodium, potassium and sucrose concentrations shown at the top (six or more lenses per medium, mean ± SE); Methionine incorporation during the 19-24th hrs of incubation in similar media (four lenses per medium, mean ± SD); *$P < 0.01$; **$P < 0.02$; $^*P < 0.05$. 

Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933338/ on 06/24/2017
Effects of Lowered Lens Potassium

During the development of sugar cataracts the [K]i equals about 70 meq/kg of lens water. In order to determine the effects of this concentration of potassium on protein synthesis, three additional media were used (Fig. 3). Medium A was used as a control. In one medium 60 mM of choline chloride was substituted for NaCl in medium A; in a second medium 25 mM galactose was added to medium A; and in a third 60 mM of NaCl was removed from medium A. When lenses were incubated for 24 hrs in these media, the K_i was in the range of 60–70 meq/kg of lens water. The incorporation of 35S-methionine into lens crystallins as indicated by the scans of autoradiographs of SDS-PAGE was equal to 15% or less of the control level. This seemed to be independent of the degree of lens swelling.

Prevention of a Depressed 35S-Methionine Incorporation

As a means of assessing the possible role of [K]i and amino acids, the three modified media described in the previous section (Fig. 3) were modified further by the addition to each of 44 mM of KCl or 25 mM of a mixture of amino acids resembling a casein hydrolysate. These additions increase the osmolality but have relatively little effect on the volume because the increase in external concentrations is countered by an increase in potassium or of amino acids within the lens. The concentration and specific activity of methionine was the same in each set of media. The results are shown in Figures 4–6. In each instance lenses incubated in media that were fortified with additional potassium had normal [K]i levels, whereas the [K]i levels in lenses incubated in media fortified with amino acids were unchanged. In media containing choline (Fig. 4) or galactose (Fig. 5) or, as noted earlier, sucrose (Fig. 2), the presence of additional potassium in the medium did not increase 35S-methionine incorporation. In the absence of such additions (in medium B [Fig. 2] or in 185 mOsm hypotonic medium [Fig. 6]) the presence of additional potassium enhanced 35S-methionine incorporation.
ration. Lenses incubated in the three media modified by the addition of choline (Fig. 4) or galactose (Fig. 5) or by the removal of NaCl (Fig. 6) incorporated more \(^{35}S\)-methionine if amino acids were added to the media.

**Discussion**

The results support the concept that protein synthesis is depressed in lenses from rats that have been on a galactose diet for six days and are consistent with the proposal\(^2\) that this deficiency is related to an inadequacy in translation. Translation might also be inhibited by a low concentration of potassium. During cataractogenesis, \([K]\) is about 70\% of normal,\(^9\) and it has been suggested that a low \([K]\) may be a factor in cataractogenesis.\(^16,27\) This is not supported by the present results.

In the absence of galactose, choline, and sucrose from the media, the pattern of protein synthesis parallels the pattern of \([K]\), levels in the lens when the incubation media and conditions are constant in other respects. Medium E (Figs. 1, 2) does not represent an exception inasmuch as a comparable hypotonic medium with a low \([K]\) was not run in the initial series. However, in the presence of galactose, choline, and sucrose in the media, a higher than normal level of \([K]\) is not associated with an elevation in protein synthesis. Therefore, it seems probable that some factor other than \([K]\) has a more direct influence on protein synthesis. The size of the amino acid pool, for instance, provides a better correlation with protein synthesis than the level of \([K]\) (Fig. 2). The fact that protein synthesis is greater in lenses incubated in media fortified with amino acids, even though the \([K]\), is low, lends further support to the idea that changes in the size of the amino acid pool rather than changes in the potassium concentration are responsible for changes in protein synthesis.

Thus, if the deficiency in protein synthesis that is noted during the formation of sugar cataracts is responsible for the final loss in the semipermeable characteristics of lens membranes, then a decrease in the size of the amino acid pool following lens swelling may be taken as a tentative step in the etiology of nuclear cataracts. The amino acid pool and protein synthesis are depressed in lenses incubated in a medium in which swelling occurs (Medium E, Figs. 1, 2) whereas the amino acid pool and protein synthesis are relatively normal if swelling is prevented by the
presence of sucrose in the medium (Medium D, Figs. 1, 2). This is consistent with earlier findings.\textsuperscript{8}

**Key words:** lens, cataracts, galactose, potassium, amino acids, protein synthesis

**References**