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MASTER

BIOCHEMICAL STUDIES ON THE OCULAR LENS
IN RELATION TO CATARACTOGENESIS

PROGRESS REPORT #15

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V. Everett Kinsey and V. N. Reddy
Institute of Biological Sciences
Oakland University
Rochester, Michigan 48063

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PROGRESS REPORT
Section 1

IONIC TRANSPORT IN THE OCULAR LENS

A major objective of this contract is to learn more about the mechanisms by which substances move into and out of the normal lens as well as those undergoing cataractous changes. Advantage has been taken of lens culture techniques developed by our group under an earlier contract with the AEC to study transport quantitatively under environmental conditions where the variables may be controlled separately.

Previous investigations led us to postulate that the lens functions like a pump-leak system in which active transport across the epithelium (pump) is balanced by passive diffusion, chiefly across the posterior capsule (leak). Recently a more elaborate hypothesis of ion movement in the lens was formulated* which was based on classic enzyme kinetics and diffusion theory, and used as a basis for a theoretical model of the pump-leak system. The model takes into account the effect of concentration of both substrate and inhibitor on the pump, and the effect of the electric as well as the chemical gradient on the leak, thus permitting conclusions to be drawn concerning the nature of the mechanisms responsible for both influx and efflux as well as the permeability properties of the bounding membranes of the lens.

Observations made of the movement of radio isotopes of potassium, rubidium, and cesium into and out of the lens in the presence of varying concentrations of nonlabeled species of each ion were shown to be accurately described by the model**. Moreover, values calculated for the parameters

* Ref. 2, Triennial Report

** Ref. 7, Triennial Report

governing the pump support the conclusion that these cations are actively transported into the lens by a carrier-mediated system probably identical or closely related to Na-K ATPase, which has a single active site.

Electric forces, presumably arising chiefly from active transport of sodium out of the lens, have a significant effect on the transport of the cations studied, increasing influx by about 15 per cent and decreasing efflux by over 60 per cent. The permeability coefficients of potassium, rubidium, and cesium under physiologic conditions were 0.95, 0.56, and 0.24 hr.⁻¹, in that order, values which are consistent with the belief that permeability of the lens for these ions is dependent upon absorptive rather than frictional forces, i.e. on the ionic field strength of the membrane that limits diffusion. The values are incompatible with any model based on purely structural considerations of the lens membrane in which transport is assumed to occur by sieving through hydrated pores.

Some years ago (AEC Contract No. C00152-57) we showed that thallium accumulates rapidly in cultured rabbit lenses reaching concentration levels greatly in excess of those present in the bathing medium. Increasing evidence for the biochemical similarity of the thallous ion and potassium ion along with more detailed knowledge of the mode of transport and diffusion of potassium, as reported above, prompted us to investigate in depth the kinetics of thallium (Tl⁺) transport in the lens, particularly as it relates to that of the alkali cations.

The parameters describing accumulation and efflux of thallium were evaluated by fitting theoretical curves to experimental data showing the movement of ²⁰⁴Tl into and out of cultured rabbit lenses in the presence of various concentrations of nonlabeled thallium, assuming that movement

occurs in accordance with the mathematical model of the pump-leak system already described. Values for the apparent Michaelis-Menten constants and maximum velocity of the carrier concerned with active transport of thallium compared with those of potassium, rubidium, and cesium are shown in Table I.

The coefficient for active transport K_p , for the pump, under physiologic conditions, is 2.25 hr.^{-1} and for the leak K_d , 0.105 hr.^{-1} , both about three times that for potassium. The identity of the carrier system for thallium with that of the alkali cations was established by cross saturation experiments. The apparent affinity of thallium for the carrier is approximately seven times that of potassium and rubidium and almost 25 times that for cesium, whereas the magnitude of V_{max} is about one quarter that for the alkali cations.

Evidence from experiments in which ouabain was employed to inhibit accumulation of all four cations suggests that the active transport system is dependent upon the action of the enzyme Na-K ATPase.

The permeability of the lens membranes to thallium, relative to the alkali cations, is unrelated to ion size, indicating that, like the latter, permeability is dependent upon absorptive rather than frictional forces.

Toxicity of thallium is manifest by opacity which develops in all lenses that have accumulated thallium to levels of about 30 mM.

The results of these studies suggested the feasibility of using the electron dense thallium as a molecular marker for potassium in electron microscopic studies.

BIOELECTRIC PHENOMENA IN THE LENS

A device in which measurements of the translenticular current, potential, and resistance of the lens can be measured under short and open-circuited conditions has been described in previous reports. This apparatus has been modified (Fig. 1) by making provision for inserting a glass microelectrode into the center of the lens as it forms a barrier between two cells, thus making it possible to determine the electric parameters across the anterior and posterior surfaces separately as well as across the whole lens.

The modified apparatus was tested by determining the responses of the anterior and posterior surface potential differences of four lenses to varying imposed translenticular voltages (by means of a voltage clamp apparatus). The potentials along with the translenticular current are shown in Fig. 2. The response of potential differences to clamped voltage is seen to be linear over most of the range measured and can be used to determine the resistance of each part of the lens. This is because the change in clamp voltage is equal to the sum of the change in current times the resistance for each part of the lens.

The results of a number of experiments on rabbit lenses maintained at approximately 37°C under varying circumstances with respect to the integrity of the capsule and epithelium are shown in Tables II to V where IA and IP signify that measurements were taken between the interior of the lens and the fluids bathing the anterior or posterior sides, respectively. Inside of lens is always negative.

The circumstances of a translenticular voltage indicates that the lens is bioelectrically asymmetric. The potentials across the two surfaces as measured

by the new microelectrode studies confirm the postulated asymmetry (Table II). Thus it was observed that the open circuit potential across the anterior surface is -41 mv, or approximately three times that across the posterior surface, viz. -16 mv, and the translens potential corresponds to the difference between the two. The short circuit potential is approximately equal to the translens open-circuited potential. The current flow is 11 1/2 microamps in the direction posterior to anterior across a resistance of 2000 ohms. Studies on decapsulated lenses show that the difference in magnitude of the potentials between the two surfaces is due to the epithelium, since removal of the anterior capsule and epithelium (Table III) decreases the translenticular voltage effectively to zero and reduces the potential across the anterior surface to roughly the same level as that across the posterior surface.

The above observations are consistent with work previously reported which demonstrates the presence of ionic pumps in the lens epithelium*. The relatively small potential difference across the posterior surface and across the decapsulated anterior surface is probably due to the fiber membranes since the intralenticular electrode is in some kind of "fiber pool." The small volume of extracellular space in the lens precludes the possibility that the electrode is in this compartment.

* Kinsey, V.E., and Reddy, D.V.N.
Studies on the crystalline lens.
XI. The relative role of the epithelium and capsule in transport.
Invest. Ophthalm. 4:104-116 (1965)

Studies on lenses decapsulated on one side only show that posterior decapsulation decreases resistance and greatly increases current. If indeed the posterior transcapsular potential arises from the lens fibers rather than the capsule, the decrease in potential after decapsulation may result from damage to the fiber membranes. This would likewise depress any current generated by the fibers in a posterior direction and account for the rise in translens current.

Removal of the capsule by digestion with collagenase for three hours results in less pronounced changes in electric phenomena (Table VI) and supports the belief that manual decapsulation injures the fiber membranes.

The fall in resistance is ascribed to changes that were shown in other experiments to occur in the lens as a result of preculture, rather than to removal of the capsule.

The effect of two potent sodium transport inhibitors, ouabain and amiloride (3, 5 diamino-6-chloropyrazinoylguanidine) on the electric parameters of the lens were determined and the results reported in Table VII in comparison with appropriate controls.

Both amiloride and ouabain are seen to decrease the open circuit voltage across the anterior surface but have little or no effect on the posterior surface. Both poisons reduce the translens potential and the translens short circuit current. The data in Fig. 3 show that the effect of ouabain on the short circuit current is not immediate but requires approximately two hours to reach a maximum. They also indicate that the maximum effect is reached at approximately 10^{-5} M concentration. No analogous data on the time course for the effect of amiloride are yet available.

BIOELECTRIC POTENTIAL OF EPITHELIAL CELLS

The fiber-epithelium potential and fiber potential of rabbit lenses as affected by cationic composition of the bathing media was studied earlier¹ and the results used to evaluate the parameters governing cation transport⁴. Ion movement affects, and in turn is also affected by, the potential differences across the epithelial cell membrane. This added feature of the electric profile across the lens was investigated during the present year.

With the aid of a micromanipulator, glass microelectrodes of approximately 0.2 microns diameter were inserted into the anterior side of lenses from which the capsule had been removed by prior incubation in KEI-4 medium containing 10 units of collagenase per ml.

The average bioelectric potential observed in two groups of lenses penetrated to different depths is shown in Table VII. The potential as determined in a single lens as a function of depth of penetration is illustrated in Fig. 4A. All the results indicate that the potential difference against a reference electrode in the medium in which the lenses were immersed is independent of the depth of penetration. Since the epithelial cells are known to be approximately 10 microns thick (Fig. 4B) it seems reasonably certain that the potential measured for lenses penetrated less than 10 microns (line 1 of the table) is that arising from a single epithelial cell. The results suggest further that resistance across the fiber membrane is low.

ION FLUXES ACROSS ANTERIOR AND POSTERIOR LENS SURFACES

Attempts were made to identify the ion or ions that give rise to the SCC* across the lens by determining the flux of radioisotopes of sodium and potassium, among others, across the entire lens under zero electrochemical gradient. Unfortunately, the fluxes were very low relative to the length of time a lens could be kept viable in the system. The radioactivity found on the cold side was always less than two per cent of that on the hot side after 12 hours. They were, therefore, thought to be unreliable, especially since the pertinent information was the difference between the unidirectional fluxes. Accordingly, fluxes across one surface at a time were determined.

The results of run-out experiments with ^{22}Na , ^{42}K , ^{86}Rb , and ^{36}Cl under zero electrochemical conditions, i.e. with the same solution bathing either surface and the translenticular voltage clamped to zero, are shown in Fig. 5. The results are shown as the percentage of the initial counts per minute in the lens remaining in the lens at the times shown. The difference in runouts into the anterior solution vs. runouts across the posterior surface is statistically significant in the case of sodium (2 hours, $p < .005$). Thus, sodium is seen to run out faster anteriorly, where it is thought to be actively pumped out, than posteriorly. While K, Rb, and Cl all appear to run out more rapidly posteriorly than anteriorly the differences are not statistically significant. If these differences were real and due to pumps actively transporting the ions into the lens it would be expected that studies of accumulation would be more sensitive indicators of surface differences.

* Short circuit current

For this reason accumulation of Rb and Cl were studied. Since rubidium is known to share a common transport system with potassium and is more readily available this ion was investigated only, along with chloride. The effect of ouabain on rubidium uptake was also determined. All of the results are shown in Table IX. The rate of uptake is expressed as the concentration of isotope in lens water at the end of the experiment divided by the initial concentration of isotope in the bathing medium. Rubidium is clearly accumulated across the anterior surface of the lens more rapidly than across the posterior surface ($p < .00005$), an effect abolished by the presence of ouabain. Chloride appears to accumulate in the lens equally across both surfaces.

The effects of ouabain (10^{-4} M) on the fluxes of Na were also examined. First, Na efflux was examined in relation to ouabain in the anterior bathing solution only, then in the posterior solution only, and finally in both the anterior and posterior media. These results are shown in Figure 6, along with Na efflux in the absence of ouabain, for comparison. Ouabain is thus seen to exert an effect only on the anterior surface efflux, and there only when it is present in the anterior bathing solution. The specific effect of ouabain on the Na efflux is seen to be a decrease in the rate of efflux across the anterior surface. This is consistent with the concept of a ouabain-sensitive Na pump, ejecting Na from the lens at the anterior surface.

The difference in efflux of sodium and influx of potassium* under zero electrochemical gradient could be due to either differences in the permeability of the two surfaces of the lens for these ions or the presence of ion pumps for one or both substances. The observation that the difference in flux were both abolished in the presence of ouabain, in the absence of any apparent effect of ouabain on efflux of either ion posteriorly, suggests that the decrease in anterior efflux of sodium and influx of potassium can be ascribed to inhibition of pumps for these ions rather than to an effect of ouabain on the permeability of the anterior barrier.

The evidence is that sodium is actively pumped out of the lens, and potassium and rubidium are actively incorporated into it, both processes taking place at the anterior surface in the epithelium and that the trans-lenticular voltage arises from the active transport of these ions and perhaps others. The data are insufficient to calculate the percentage contribution of sodium flux to the SCC.

Chloride, on the other hand, appears to move in a strictly passive manner; it does not appear to be either actively pumped itself or directly linked to the active transport of another ion.

The data shown in Table X have a further bearing on the nature of the movement of chloride across the surfaces of the lens.

* As determined with rubidium (Table IX).

The increase in current and potential when sulfate is substituted for chloride indicates difference in permeability of lens membranes to sulfate.

The persistence of a small current and potential when lithium is substituted for sodium suggests that lithium is transported by the sodium pump but less efficiently.

Removal of chloride and both sodium and chloride failed to alter current or potential suggesting that there is no chloride pump acting in either direction.

FIGURE 1

Lens in voltage-clamp apparatus showing position of voltage and current electrodes.

FIGURE 2

EFFECT OF OUABAIN ON SHORT-CIRCUIT CURRENT IN THE LENS

FIGURE 3

FIGURE 4-A

SINGLE LENS EPITHELIAL

Potential vs. Depth

FIGURE 4-B

FIGURE 5

PER CENT OF INITIAL LENS RADIOACTIVITY THAT HAS RUN-OUT VS. TIME

FIGURE 6
EFFECT OF OUABAIN ON ^{22}Na RUN-OUT VS. TIME

PER CENT INITIAL RADIOACTIVITY REMAINING IN LENS

TABLE I

Apparent Michaelis-Menten constants and maximum velocity of the carrier concerned with active transport of thallium in rabbit lenses compared with those of potassium, rubidium, and cesium.

Saturating Ion	K _m nM	V _{max} μmoles/hr./lens
Tl	0.15	0.375
K	1.1	1.0
Rb	1.1	1.0
Cs	3.6	1.0

TABLE II
INTACT - 33 Cases Each

	Open Circuited mv	Short Circuited mv	Current μ A	Resistance Ω
IA	-41 ± 6.9	-28 ± 7.6	--	--
IP	-16 ± 7.9	-28 ± 7.6	--	--
Translens	25 ± 6.1	0 ± 0	11.5 ± 5.5	2000 ± 500

TABLE III
DECAPSULATED ANT. SIDE ONLY

	Open Circuited Potential Diff. mv	Short Circuited Potential Diff. mv	Current μ A	Resistance Ω
IA	-8 ± 4.6 (6)	-9 ± 4.5 (6)	--	--
IP	-12 ± 4.4 (6)	-10 ± 4.8 (6)	--	--
Translens	-4 ± 3.4 (6)	0 ± 1.6 (6)	-1 ± 3.3 (8)	800 ± 115 (7)

TABLE IV
DECAPSULATED POST. SIDE ONLY

	Open Circuited mv	Short Circuited mv	Current μ A	Resistance Ω
IA	-32 ± 2.3 (6)	-14 ± 2.8 (6)	--	--
IP	-6 ± 2.0 (6)	-14 ± 2.8 (6)	--	--
Translens	$+29 \pm 4.2$ (6)	0 ± 0 (6)	28 ± 5.0 (8)	1080 ± 200 (8)

TABLE V
DECAPSULATED COMPLETE

	Open Circuited mv	Short Circuited mv	Current μ A	Resistance Ω
IA	-17, -9	-20, -7.5	--	--
IP	-18, -10	-20, -7.5	--	--
Translens	-1, -1	0, 0	-3, -4	540, 520

IA - interior to anterior side; IP - interior to posterior side

TABLE VI

Lens precultured in collagenase 3 hours to remove capsule

	Open Circuited mv	Short Circuited mv	Current μ A	Resistance Ω
IA	$-36 \pm (10)$	$-20 \pm (9)$	--	--
IP	$-12 \pm (10)$	$-20 \pm (9)$	--	--
Translens	$24 \pm (10)$	0	$20 \pm (10)$	$1200 \pm (9)$

TABLE VII
LENSES PRECULTURED 6 HOURS IN KEI-4 (CONTROLS)

	Open Circuited mv	Short Circuited mv	Current μ A	Resistance Ω
IA	-43 ± 5.0 (8)	-24 ± 4.7 (8)	--	--
IP	-17 ± 5.0 (8)	-24 ± 4.7 (8)	--	--
Translens	$+27 \pm 5.7$ (8)	0 ± 0 (8)	18 ± 2.2 (8)	1400 ± 220 (8)

LENSES PRECULTURED 6 HOURS IN 10^{-4} M AMILORIDE

	Open Circuited mv	Short Circuited mv	Current μ A	Resistance Ω
IA	-30 ± 6.7 (7)	-20 ± 6.7 (7)	--	--
IP	-19 ± 4.0 (7)	-20 ± 6.7 (7)	--	--
Translens	$+13 \pm 8.0$ (7)	0 ± 0 (7)	9 ± 2.7 (8)	1430 ± 230 (8)

LENSES PRECULTURED 7 HOURS IN 10^{-4} M OUABAIN

	Open Circuited mv	Short Circuited mv	Current μ A	Resistance Ω
IA	-17.5 ± 2.3 (6)	-13.7 ± 3.7 (6)	--	--
IP	-10.8 ± 3.5 (6)	-13.7 ± 3.7 (6)	--	--
Translens	$+6.7 \pm 3.3$ (6)	0 ± 0 (6)	2.1 ± 1.1 (8)	1537 ± 147 (8)

IA - interior to anterior side; IP - interior to posterior side

TABLE VIII

BIOELECTRIC POTENTIALS EPITHELIUM

Av. 3 Punctures Each Lens

<u>Potential</u> mv		<u>Depth</u> microns
-31.7 ± 4.7	(8)	3 to 10
-32.5 ± 5.4	(6)	11 to 150

TABLE IX

Accumulation of rubidium, with and without ouabain, and chloride after 2 hours contact with the anterior or posterior surface.

<u>Ion</u>	<u>Surface Exposed</u>	<u>Cl/Cm</u>
^{86}Rb	Anterior	$.28 \pm .09$ (6)
	Posterior	$.11 \pm .03$ (7)
$^{86}\text{Rb} + 10^{-4}$ ouabain	Anterior	$.11 \pm .05$ (6)
	Posterior	$.12 \pm .03$ (6)
^{36}Cl	Anterior	$.07 \pm .03$ (9)
	Posterior	$.10 \pm .06$ (7)

TABLE X

Effect of ionic composition of media on bioelectric phenomena.

<u>Media</u>	<u>SCC</u> <u>μA</u>	<u>Translens</u> <u>mV</u>	<u>IA</u> <u>mV</u>	<u>IP</u> <u>mV</u>
(Complete)				
Na + Cl	<u>11.5</u>	<u>25</u>	<u>-41</u>	<u>-16</u>
(Cl-free)				
Na + SO ₄	21.5	40	-43	-3
(Na-free)				
(K+Li) + Cl	3.2	7.3	-31	-24
Li + Cl	8	15	-34	-19
(NaCl-free)				
Li + SO ₄	7.8	18	-35	-19
Mannitol	1.8	6	-42	-36

All changes in electrical phenomena were reasonably reversible except in the case of lenses in mannitol.

PROGRESS REPORT

Section 2

STUDIES ON GLUTATHIONE IN THE LENS

A considerable portion of research supported by this contract in recent years has been concerned with the metabolism of glutathione in the lens and its possible role in cataractogenesis. Attention has been drawn in previous reports that it is a common feature of nearly all types of cataracts that the level of glutathione decreases in the lens. The mechanism by which glutathione is lost from the lens has never been adequately explained. Studies based on the rates of incorporation of the precursor amino acids, glutamic and glycine, suggest that the tripeptide is synthesized in the lens. Since the steady state level of glutathione remains constant through the experimental period, it is presumed that this synthetic activity is balanced by a similar rate of degradation of the tripeptide. However, to date there is no convincing evidence that the lens contains peptidases which are capable of degrading glutathione. Another possibility that glutathione may be continuously lost by leakage out of the lens has also not been substantiated. The current status of the problem of the metabolism of glutathione in the normal and cataractous lens has been summarized in a recent paper⁶.

Some of the unanswered questions are: 1) Is glutathione transported out of the lens?, 2) Is there in fact a mechanism for the breakdown of glutathione in the lens?, and 3) Does the incorporation of the constituent amino acids in glutathione in situ represent total breakdown and resynthesis of the tripeptide?

During the current year we have carried out a series of investigations in order to provide a better understanding of the aforementioned problems.

TRANSPORT OF GLUTATHIONE

Our experimental approach to test the possibility that glutathione is actively transported out of the lens was to study the rate of entry of both reduced and oxidized glutathione into the lens and attempt to interfere with the transport mechanisms. If oxidized glutathione is in fact actively transported out of the lens, its rate of entry into the lens across the anterior surface containing the epithelial cells should be lower than the posterior surface of the lens. These experiments would be analogous to the studies of the penetration of sodium ions into the lens, which is known to be actively transported out of this organ.

Preliminary studies using ^{35}S -labeled glutathione showed that much more GSH entered the lens than GSSG. One of the technical difficulties in comparing the rates of entry of reduced and oxidized glutathione in the lens is that reduced glutathione is easily oxidized so that the rate of entry of this compound is not a true measure of the transport of the reduced tripeptide. To get around this difficulty, dithiothreitol, a well known reducing agent for sulfhydryl compounds, was used in conjunction with GSH which prevented the oxidation of the compound. It was found that three times as much GSH enters the lens across the epithelial surface than the posterior part of the lens (Table I). Chromatographic analysis on an amino acid analyzer showed that much of the radioactivity in the lens was associated with GSH. Furthermore, the entry of labeled glutathione into the lens was reduced with the addition of nonlabeled glutathione, suggesting that the mechanism of transfer is a saturable one (Table II). The rate of entry of GSSG into the lens, which is considerably slower than the reduced compound appears to be un-

affected by the addition of nonlabeled oxidized glutathione. Since both labeled GSH and GSSG contain small amounts of unidentified radioactive impurities and because relatively few counts of radioactivity are observed in the lens when individual lens surfaces were exposed to medium containing oxidized glutathione, it has not yet been possible to determine to what extent the radioactivity in the lens is due to ^{35}S -labeled GSSG.

Accumulation of labeled GSH and GSSG in the lens was also studied using culture technique. The accumulation of radioactivity in the lens was considerably higher when the lenses were cultured in Tyrode's medium than in KEI-1A or 4. The ratio of the radioactivity in the lens water to that in the initial medium (CL/CM) was nearly 7 in Tyrode's medium while that in KEI medium was less than 2 (Table III). It was considered possible that some of the ions and other constituents present in the KEI medium could be catalyzing the conversion of GSH to unidentified compounds which may not be concentrated by the lens. However, the inorganic ions such as iron, zinc or magnesium which are present in KEI medium when included in Tyrode's solution, showed no effect on the accumulation of GSH by the lens. In order to investigate the integrity of GSH molecule in different media, labeled GSH was incubated in both Tyrode's and KEI medium for a period of 24 hours and the media chromatographed on an amino acid analyzer. In Tyrode's medium GSH molecule remained unchanged at the end of the incubation period whereas in the KEI medium less than 5% of total radioactivity was present in the form of GSH; also in this medium a number of unidentified compounds were formed. Thus, because of the rapid disappearance of the tripeptide from the KEI medium, less glutathione was accumulated in the lens.

As a result of these findings, all other subsequent experiments on the accumulation of radioactive glutathione in the lens were performed by culturing lenses in Tyrode's medium. The time course of accumulation of ^{35}S -labeled GSH in lenses cultured in Tyrode's medium is shown in Figure 1. At the end of 24 hours the distribution ratio (CL/CM) based on total radioactivity was approximately 7. Since these results do not represent the actual concentration ratio of GSH, if the molecule is metabolized, a number of experiments were performed in which both the lens extracts and the media after different periods of incubation were chromatographed and the radioactivity associated glutathione molecule measured, following the separation of the compound on an amino acid analyzer. The distribution ratio (CL/CM) based on GSH measurements (Fig. 1) was approximately 4 at the end of 24 hours. Thus, it appears that glutathione is concentrated by the lens against a concentration gradient - a totally unexpected finding.

The transport process for glutathione appears to be readily saturated by the addition of non-labeled glutathione to the incubation medium. Of the three constituent amino acids in the tripeptide, only cysteine was found to compete for the transport system of glutathione in the lens, suggesting that SH group may be involved in the transport of GSH (Table IV).

Other preliminary experiments indicate that the capacity of the lens to accumulate GSH is reduced in the absence of glucose from the medium and at reduced temperature.

Evidence for the breakdown of GSH in the lens

In experiments designed to measure the radioactivity associated with glutathione molecule in the lens extract and the post-incubation media it was observed that while major portion of radioactivity in the lens was due to glutathione, significant amounts of radioactive cysteine which must have been derived from the labeled glutathione was also present (Table V). Various unknown radioactive compounds were also detected following incubation of lenses in the presence of ^{35}S -labeled GSH. These compounds may be derived either from glutathione directly or from cysteine after the degradation of the peptide.

Figure 2 shows that with increasing times of incubation there is an increase in the formation of cysteine in the lens. These studies clearly suggest that the lens contains a mechanism for the degradation of the tripeptide, evidence for which was lacking hitherto. Thus, in part, the turnover of glutathione molecule in the lens must involve a total breakdown of the tripeptide.

Incorporation of cysteine into glutathione

In studies described in earlier AEC reports, mention has been made of the rates of incorporation of glycine and glutamic acid into glutathione in the lens. Since the rates of incorporation of the two amino acids were approximately the same, it was considered unlikely that the turnover of these amino acids represented exchange reactions. However, these results did not permit the conclusion that incorporation of terminal amino acids was due to total breakdown and resynthesis of glutathione in the lens.

To provide evidence for the breakdown and total synthesis of glutathione molecule from its constituent amino acids in the lens, it would be necessary to demonstrate that cysteine, the third amino acid in the peptide, is also incorporated along with glycine and glutamic acid. A major difficulty in studying the turnover of cysteine in glutathione molecule is that the amino acid is unstable in the culture medium with a half life of approximately twenty minutes. During the past year we were able to get around this difficulty by including dithiothreitol in the medium along with labeled cysteine. Under these conditions, it was observed that significant amounts of labeled cysteine which entered the lens were incorporated into lens glutathione. In order to determine the turnover rate of this amino acid in glutathione, it is necessary to establish the level of free cysteine pool in the lens. Since cysteine is eluted in the same position as glutamic acid, it has not been possible to estimate the concentration of cysteine in the lens. The estimation of turnover rate of this amino acid in GSH must, therefore, await development of a suitable method for the chromatographic separation of cysteine from other amino acids. Nevertheless, the results thus far indicate that all of the three constituent amino acids of GSH are incorporated into the tripeptide and that ^{it} is broken down completely and resynthesized within the lens.

THE ROLE OF INOSITOL IN MEMBRANE TRANSPORT

Inositol is an important component of biological membranes and is present in unusually high concentrations in most ocular tissues. Its concentration decreases in the lens of galactose-fed and diabetic animals. Inositol has been implicated in the transport of certain amino acids across membranes.

For this reason, extensive investigations concerning the mechanisms which may be responsible for maintaining high concentration of this substance in the lens and intraocular fluids were undertaken. The results of these investigations were described in last year's report^{and} have been published^{4,5}.

The current concept regarding the role of inositol in transport involves the turnover of phosphoinositide in the membrane lipids. While the exact mechanism is not understood, this substance has been implicated in the transport of ions and possibly other substances. Previous studies in this laboratory have shown that amino acids are actively transported into the lens and that the mechanism of the transport of these compounds is impaired in diabetic lenses. Since diabetes has been shown to affect lipid composition of other tissues, it was considered of interest to investigate the possible relationship between the impairment of amino acid transport in diabetic lens and changes in the phospholipid composition of this tissue. Because lens is avascular and derives many of its constituents from the aqueous humor, changes in the composition of lens could conceivably reflect changes in the aqueous humor. For this reason, phospholipid composition in the aqueous humor of normal and diabetic animals were also studied. Details of this investigation are summarized in preprint #7 which will appear in Experimental Eye Research. A summary of the findings follows.

The phospholipid composition of the aqueous humor, plasma and ocular lens of normal and diabetic rabbits was determined using thin layer chromatography. The presence of this class of compounds in the aqueous humor has been demonstrated for the first time. Lysophosphatidyl choline, sphingomyelin,

and phosphatidyl choline constitute the main fractions. The phospholipids of the plasma were lysophosphatidyl choline, sphingomyelin, phosphatidyl choline, phosphatidyl inositol and phosphatidyl ethanolamine. Lens, in addition to the phospholipids present in the plasma also contained phosphatidyl serine.

In the aqueous humor of diabetic animals, the concentration of lysophosphatidyl choline, phosphatidyl choline and some unidentified lipids were found to be reduced while that of sphingomyelin increased. In contrast, the concentrations of nearly all of the plasma phospholipids, including that of phosphatidyl choline, were observed to increase. The phospholipid composition of the lens in normal and diabetic animals was essentially similar, except that in the latter the concentration of phosphatidyl inositol was lower.

Although a direct relationship exists between the decreased level of phosphoinositide in diabetic lens and the impairment of amino acid transport in this tissue as was shown in our previous study, further data, particularly on the incorporation of inositol and the phosphoinositide and the turnover rate of the phosphatide as affected by diabetes, are needed to establish a possible role of this phospholipid in the transport of amino acids in the lens.

THE ROLE OF SORBITOL PATHWAY IN THE CILIARY BODY IN RELATION TO ACCUMULATION OF AMINO ACIDS IN THE AQUEOUS HUMOR OF ALLOXAN DIABETIC RABBITS

Previous studies in this laboratory have shown that the rates of accumulation and the steady state concentration of amino acids in the lens and aqueous

humor are reduced in diabetic animals. The reduced capacity of diabetic lenses to accumulate amino acids has been ascribed to both a decrease in active transport and an increase in the permeability of the surface membranes of the lens as a secondary result of high concentration of sorbitol. van Heyningen has shown that sorbitol accumulates in lenses when the animals are made diabetic by alloxan administration. Since the lens membranes are relatively impermeable to sugar alcohols, the accumulation of these compounds leads to osmotic swelling of the lens, thus affecting the permeability of the membranes. If such an accumulation of sorbitol were to occur in the ciliary epithelial cells, it could account for the depressed level of amino acids in the aqueous humor of diabetic animals.

Studies aimed at testing the hypothesis were completed during the current year and the results have been published⁸. The findings of this investigation are summarized below.

The activity of the enzymes aldose reductase and polyol dehydrogenase associated with sorbitol pathway were determined in the rabbit ciliary body and compared with the activity of these enzymes in the lens and the retina, a tissue which has the same embryological origin as the ciliary body. Despite the presence of the two enzymes, no sorbitol could be detected in the ciliary body of diabetic rabbits. These results suggest that the accumulation of sorbitol in the ciliary body and presumably in the ciliary epithelial cells, which are responsible for transport, is not a contributory factor in the reduction of amino acid levels observed previously in the aqueous humor of these animals. The reasons why sorbitol does not accumulate in the ciliary body of diabetic rabbits despite the presence of the necessary enzyme systems are discussed.

TABLE I

Influx of $G^{35}SH$ in cpm/lens when a single surface is incubated for 1 hr. in Tyrode's medium containing 10^5 cpm/ml.

Anterior	Posterior	<u>Anterior</u> <u>Posterior</u>
501	145	3.46
492	132	3.73
267	114	2.34
317	106	2.99
259	108	2.41
<u>367 ± 107</u>	<u>121 ± 15</u>	<u>2.99 ± 0.55</u>

TABLE II

Influx of $G^{35}SH$ in cpm/lens when a single surface is incubated for 1 hr. in Tyrode's medium containing 10^5 cpm/ml and 5 mM nonlabeled GSH.

Anterior	Posterior	<u>Anterior</u> <u>Posterior</u>
129	117	1.10
109	145	0.75
106	71	1.49
108	60	1.80
146	73	2.00
<u>120</u> $\pm$ 16	<u>93</u> $\pm$ 32	<u>1.43</u> $\pm$ 0.46

TABLE III

Accumulation of ^{35}S -labeled GSH in rabbit lenses cultured for 24 hours in various media. Results are expressed as a ratio of the total radioactivity in the lens water to that in the initial medium of incubation.

Medium	CL/CM (Average value)	No. of ex- periments
Tyrode's	7.3	21
KEI-1A	1.7	6
KEI-4	1.7	2
Inorganic salt solution present in KEI medium	3.5	4
Tyrode's with bicarbonate content equal to KEI	4.0	3
Tyrode's with iron content equal to KEI	6.7	2
Tyrode's with Zn content equal to KEI	8.3	2
Tyrode's with Mn content equal to KEI	7.5	2

TABLE IV

Influence of the addition of various non-labeled compounds (5mM) on the accumulation of ^{35}S -labeled GSH as measured by total radioactivity in lenses cultured for 24 hours. The results are expressed as percentage of contralateral controls incubated in Tyrode's solution containing labeled GSH only

Compound added	Accumulation ratio (% of control $\pm$ range)	No. of experiments
Control	100	
L-Ascorbic acid	116 $\pm$ 13	3
Glycine	95	2
Glutamic acid	110	2
Cysteine	32 $\pm$ 5	6
GSH	15 $\pm$ 6	5

TABLE V

Percentage distribution of radioactivity in lens extracts and post incubation media after various periods of incubation in Tyrode's solution containing ^{35}S -labeled GSH.

Radioactive Peak No.	Compound	Lens				Media			
		3 hr	6 hr	12 hr	24 hr	3 hr	6 hr	12 hr	24 hr
I	U	1.75	1.5	2	1	3	3.5	5.5	1.2
II	U	2.5	1.25	2	1.5	-	-	6	1
III	U	4	1.4	2	1	-	1.7	2.2	-
IV	U	-	1.7	-	1.5	1	6	-	1.0
V a	GSH	78	73	66	53	89	88	80	97
V b	U	-	-	-	-	6	-	-	-
VI	GSSG	8	12	-	-	0.5	1	3	-
VII	Cysteine	0.5	3	15	25	1	0.5	1	-
VIII	Cystathionine	-	-	3.2	2	-	-	10	-

U: unidentified.

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