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Lens in Relation to Cataractogenesis," February 1, 1972 - January 31, 1973
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Application to
U. S. ATOMIC ENERGY COMMISSION

for

BIOCHEMICAL STUDIES ON THE OCULAR LENS IN
RELATION TO CATARACTOGENESIS

Continuation of AT(11-1)-2012

For the Period
February 1, 1973 to January 31, 1974

Principal Investigator:

V. Everett Kinsey

Director of Research Services

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BIOCHEMICAL STUDIES ON THE OCULAR LENS IN
RELATION TO CATARACTOGENESIS

Progress Report

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February 1, 1972 to January 31, 1973

PREPARED FOR THE U. S. ATOMIC ENERGY COMMISSION UNDER CONTRACT No.
AT(11-1)-2012

ABSTRACT

Transfer coefficients describing the rate of accumulation and net efflux of sodium in rabbit lenses were evaluated on the basis of a theoretical model of a pump-leak system that treats the lens as a three compartment system: the capsule, medium and lens fibers.

Active transport of sodium from the lens apparently is responsible for the electrical current that was shown to flow across both the anterior and posterior surface of the lens. Flow anteriorly is approximately twice that posteriorly, and occurs across a larger electrical resistance.

Studies of electrical coupling between lens fiber cells lead to inconclusive results.

Transport of reduced glutathione into the lens was found to occur by a carrier mediated process that appears to share the same carrier site as that for cysteine. Evidence was obtained that peptides in general may be transported into the lens by processes other than diffusion.

The complex nature of the relation between the transport of amino acids and inositol to that of sodium and potassium were demonstrated and critically analyzed.

PROGRESS REPORT

Section I.

IONIC TRANSPORT IN THE OCULAR LENS

Sodium

Concentration of sodium in normal lenses is only about 15% of that in intraocular fluids. For the most part, the concentration gradient is maintained by a Na-K ATPase activated pump that transports sodium out of the lens, possibly coupled with the influx of potassium. Entrance of sodium occurs passively down both the electric and chemical gradients created by the pump. Attempts have been made in this laboratory over the past several years to evaluate the transfer coefficients of the parameters, K_p and K_d , that govern sodium movement using the same theoretical model of a pump-leak system that was employed to determine analogous coefficients for other cations (K, Rb, Cs, Tl). Although repeated efforts were made it was not possible to fit data describing both rate of accumulation and net efflux of sodium with a single pair of rate constants. In view of the key position occupied by sodium in determining ion and water balance in the lens the problem was extensively reinvestigated during the current year.

The theoretical model (see previous AEC reports) employed to determine the parameters was based on a two compartment system, lens and medium. Unlike the situation with potassium, a large proportion of the sodium in the lens is contained in the highly porous capsule where it exchanges much more rapidly with sodium in fluids bathing the lens than does that fraction of the sodium in the cortex or nucleus. It was necessary therefore to consider this fraction

separately from that residing within an ion-limiting membrane (fibers) when describing rates of accumulation and efflux of this cation in the whole lens. For this purpose, a mathematical model was developed that treated the lens as a three compartment system: the medium, capsule, and lens fibers.

Rate of accumulation and net efflux of ^{22}Na was determined in cultured rabbit lenses and attempts were made to fit the data with theoretical curves calculated on the basis of the mathematical model. As in earlier experiments, no single pair of values for K_p and K_d would fit both sets of data. Moreover, it was observed that the ratio of concentration $\frac{C_L}{C_M}$ under steady state conditions was considerably above that found for non-radioactive sodium in lenses immediately after removing them from the globe.

Further experimentation showed that the reason both sets of flux data could not be fitted with the same value for the transport coefficients was related to differences in the total length of time lenses were cultured when determining rates of accumulation and net efflux. The former were measured in lenses immediately after removal from the eye, whereas the latter were determined only after they were preloaded with ^{22}Na for 18 hours. The rate of diffusion of ^{22}Na was therefore redetermined, but only after lenses were precultured for a period of three hours. Under these circumstances, fits were obtained to both influx and efflux data with the same values of K_p and K_d throughout the whole time course. One difficulty still remained, however, since the sodium concentration at steady state was still significantly above normal. This finding suggested that while injury associated with removing

the lens from the eye is repaired, healing is incomplete.

Among other changes employed in technique of removing the lens from the eye, zonules were cut much closer to their origin in the ciliary body. This procedure resulted in less injury to the lens as evidenced by a significant reduction in the rate of accumulation of sodium during the first several hours. More importantly, the rate, which became constant after about three hours, appeared to be identical with that prevailing in vivo since the ratio of concentration lens/medium at steady state is essentially identical with that obtained for non-radioactive sodium from noncultured lenses, viz., $0.13 \pm .023$. Rates of accumulation of ^{22}Na determined in lenses removed by the modified technique, and precultured for three hours, could be fitted by the same pair of rate constants that fit net efflux data, viz., $K_p = 0.58 \text{ hr}^{-1}$ and $K_d = .010 \text{ hr}^{-1}$. These values are believed to correspond to those in the living eye.

The results support the conclusion that variations in the rate of diffusion were responsible for observed differences in rate of accumulation soon after removal from the eye and demonstrate that permeability of ion restricting membranes of the lens are readily affected by trauma. Additional observations suggest that the mechanisms responsible for active transport of sodium too, may be sensitive to trauma, but less so than those affecting permeability.

Availability of values for the rate constants made it possible to compare the movement of sodium in the lens with that of potassium. The rate at which nonlabeled sodium is actively transported out of lens fibers is equal to the product of K_p (0.58 hr^{-1}), $[\text{Na}]_F$ (11.6 mM) and

V_F (0.186 ml), or 1.25 $\mu\text{moles/hr/lens}$. The corresponding rate of active transport of potassium into the lens, calculated for a value $K_p = 0.82 \text{ hr}^{-1}$ found in previous studies under similar conditions, is 0.82 $\mu\text{moles/hr/lens}$. Thus, three sodium ions are actively transported out for each two potassium ions transported into the lens, a ratio identical with that found in red blood cells. No evidence was found to indicate that the efflux of sodium is coupled to the influx of potassium.

The relative rate of passive diffusion of sodium which is identical with that of the larger ion cesium, is only about one fourth that of potassium ($K_d = .039 \text{ hr}^{-1}$).

Details supporting this brief description of the study of sodium transport are given in the accompanying preprint #1 of a paper to appear in Experimental Eye Research.

BIOELECTRIC PHENOMENA IN THE LENS

The results of a number of experiments concerned with bioelectric activity of rabbit lenses were described in some detail in last year's AEC Progress Report. Electrical measurements were made on lenses fixed between two plastic cells across which voltage could be clamped to zero by means of a voltage clamp apparatus. The lens was found to be asymmetric bioelectrically, with open circuit voltage across the anterior surface (-41 mv) approximately three times that across the posterior surface (-14 mv), and the current flow under short circuit conditions equal to about 12 micro amps in a posterior to anterior direction.

The electrical asymmetry was thought to result from net differences in the rates of cation fluxes across the anterior and posterior surfaces of the lens. Thus the rate of sodium efflux anteriorly was shown to be greater than posteriorly, a difference not compensated for by flux of potassium. However, no information was available concerning the separate short circuit currents across the lens surfaces or their relative conductances.

To make these measurements it was necessary to modify the voltage clamp apparatus so that the voltage could be clamped to zero across the anterior or posterior surfaces separately. This was done by introducing a Ag-AgCl electrode into the lens at the equator parallel to the glass micro electrode used to measure voltage. The results of a number of experiments performed with this apparatus are shown in Table I. The current across the anterior portion of the lens is seen to be twice that across the posterior portion, 33 vs. 17 micro amps.

The translens current is thus the net difference between the two, and directed anteriorly.

The resistance is also appreciably higher across the anterior portion of the lens (1150 ohms) than across the posterior portion (890 ohms), indicating that the permeability of the surface membranes on the anterior side is less than that of the membranes on the posterior surface, due perhaps, to the presence of the epithelium anteriorly.

The current directed outward suggests that the fiber membranes, as well as the epithelial cells, are capable of extruding sodium from the lens, however, the mechanism responsible appears to be different since the net efflux of sodium across the anterior surface is markedly reduced by ouabain whereas that from the posterior side is not (see AEC Progress Report, 1971). The proportion of the total in-out current in the lens occurring anteriorly (66%) is nearly the same as the fraction of the total net efflux of sodium shown in recent experiments to be ouabain-sensitive (60%). The small difference may result from the retarding effect of the bioelectric potential (42 mv) on movement of sodium from lenses, since in the latter experiments the potential was not clamped to zero during the course of measuring net efflux (Preprint #1).

While more evidence will be required to draw final conclusions, it is an attractive hypothesis to suggest that approximately two thirds of the sodium actively transported out of the lens takes place by a carrier-mediated process dependent on, if not identical with, Na-K ATPase across the epithelium and one third across the posterior fiber membranes by a mechanism, or mechanisms, as yet unknown.

Electrical Coupling and Cell Junctions in the Lens.

Electrical coupling is a low resistance pathway by which ions move between the cytoplasm of a cell and the cytoplasm of adjacent cells in a tissue. Thus, coupling between cells would cause a tissue to behave as an electrical syncytium explaining the absence of a potential gradient and the rapid distribution of potassium ions observed in rabbit lenses.

Experiments were performed measuring two parameters: the input resistance and the coupling coefficient of lens fiber cells which are dependent on electrical coupling. The input resistance of a cell is defined as the ratio of the change in membrane potential to the magnitude of current passed between the cytoplasm of the cell and the external medium. The input resistance of a coupled cell is lower than the input resistance of an uncoupled cell because in the uncoupled cell ions carrying the current can only move across the plasma membrane of a single cell, while for the coupled cell ions can move between adjacent cells and then across the plasma membranes of these cells as well. Since depolarization currents are known to uncouple cells (Socolar, S. S.: Discussion, ICER Symposium, Charleston, S. C., 1972), the input resistance measured with a depolarizing current should be greater for a coupled cell than for one measured with a hyperpolarizing current.

The input resistance was measured in rabbit lenses with double barreled micro electrodes having tip diameters of 3-4 μ , using both a depolarizing and hyperpolarizing current. The respective resistances found were $640 \pm 160 \text{ K}\Omega$ and $390 \pm 170 \text{ K}\Omega$. This difference is greater

than would be expected from rectification of the plasma membrane, and therefore is believed to indicate the presence of cell coupling in the lens.

The coupling coefficient is defined as the ratio of the change in membrane potential of a cell to the change in membrane potential of another cell in the tissue, when a current is passed between the second cell and the external medium. The coupling coefficient is a more direct measurement of cell coupling than the input resistance. It is also a more technically difficult measurement to make because it involves three electrodes, two placed in the same cell (a double barreled electrode), and a third one in a second cell. Ideally the second cell should lie adjacent to the first cell since the coupling coefficient decreases rapidly with the number of intervening cells in most tissues. In the present experiments it was only possible to get the electrode tips within 40 μ (about 4 cells apart) and no evidence of coupling was found. Since some evidence of coupling should have been present at even this distance if the cells were coupled, it can be concluded that under these experimental conditions the lens fiber cells are not coupled. However, Socolar states that minimal trauma can uncouple cells; it is possible, therefore, that the shearing forces produced by inserting two electrodes at close distances in the lens could have uncoupled the fiber cells.

the fiber cells.

Evidence obtained thus far concerning the question of whether lens fiber cells are electrically coupled is inconclusive. Thus, the experiment in which the input resistance was determined with depolarizing

and hyperpolarizing currents indicated the possibility of electrical coupling, while those in which the coupling coefficient was determined directly failed to demonstrate electrical coupling, possibly for technical reasons.

TABLE I

Mean potential, current, resistance, and conductance of non-cultured rabbit lenses when short circuited across the anterior or posterior surfaces. (Lenses impaled with both glass micro electrode and Ag-AgCl electrode.)

	Condition	
	Short Circuit (Across Ant. Portion)	Short Circuit (Across Post. Portion)
<u>Potential</u>		
Translens	19 ± 3 mv (24)	30 ± 12 mv (23)
Across ant. portion	0 mv	30 ± 12 mv (23)
Across post. portion	19 ± 3 mv (24)	0 mv
<u>Current</u>		
Across ant. portion	33 ± 11 μ A (34)	—
Across post. portion	—	17 ± 11 μ A (35)
<u>Resistance</u>		
Across ant. portion	1150 ± 300 ohms (34)	—
Across post. portion	—	890 ± 400 ohms (35)
<u>Conductance</u>		
Across ant. portion	$9.5 \pm 2.7 \times 10^{-4}$ mhos (34)	—
Across post. portion	—	$13.4 \pm 5.2 \times 10^{-4}$ mhos (35)

Section II.

STUDIES ON AMINO ACID AND PEPTIDE TRANSPORT

A recent line of development on the transport systems in the lens in our laboratory was to inquire whether glutathione (GSH), a tripeptide found in unusual concentration in the lens, has a special mechanism for its exit from the lens, since at the present time there is no known mechanism by which glutathione is lost from this tissue. The mechanism for transport of oxidized glutathione (GSSG) is important in view of the observations made in the red blood cell where it was shown to be actively transported out of the cell and that a similar mechanism may be responsible for the loss of glutathione from lenses. The results to date indicate that no analogous active transport mechanism exists in the lens and that GSSG may leave this tissue by simple diffusion. However, an unexpected finding was that the entry of reduced glutathione into the lens is carrier-mediated.

The mechanism of transport of GSH into the lens was further explored during the current year. The transport system appears to share the same carrier site as that for cysteine. The other two constituent amino acids are without any competitive effect, suggesting that -SH may be an important functional group.

In view of the observations that the lens is capable of actively transporting glutathione inward, we have also initiated studies to examine if peptides, in general, enter the lens by a carrier-mediated process. ¹⁴C-labeled glycyl-glycine was selected as a model compound since it is clearly separated from other amino acids and glutathione in our chromatographic system.

Studies thus far have indicated that this dipeptide enters the lens by a process other than diffusion and is concentrated against a gradient. The transport appears to be active since lack of glucose or the presence of iodo-

acetate in the media reduces the accumulation of this peptide in the lens. Its entry was also found to be reduced in the presence of glycine, suggesting that the peptide may use, in part, the same mechanism as the amino acid. In these studies the possibility that the peptide may be first hydrolyzed to its constituent amino acid at the lens surface and thus gain entry in the form of the amino acid was also considered. The results suggest that hydrolysis of the dipeptide takes place within the lens. Analyses of the media following culture of the lenses in the presence of ^{14}C -glycyl-glycine indicates that there is no radioactive glycine in the "post culture" media. Also, the carrier system responsible for the entry of labeled glycyl-glycine into the lens is saturated more readily with nonlabeled dipeptide than with equivalent amounts of free glycine (Table II).

The results clearly suggest that a variety of peptides may be transported into the lens by carrier-mediated process and may have an overlapping affinity for the transport site of one of its constituent amino acids. During the coming year the characteristics of these transport systems will be further explored.

Effect of cations and the role of Na-K ATPase

The dependence of amino acid transport on intracellular and extracellular cations has been observed in many tissues including the lens and ciliary body. However, the nature of the coupling mechanism between the transport of cations and amino acids is not understood. It has previously been shown in this laboratory that ouabain, a specific inhibitor for Na-K ATPase, not only affects cation transport, but reduces the accumulation of amino acids and inositol in the lens, suggesting that the membrane ATPase may be involved in the transport of the latter compounds. While the effect of ouabain is immediate on cation

transport, its effect on the accumulation of α -AIB was noted only after a delay of two or more hours, suggesting that the effect of cardiac glucoside on amino acid transport may have been secondary. This interpretation was found to be consistent with the finding that there was no interdependence on the transport of potassium ion or α -AIB into the lens, i.e. the transport of neither potassium nor α -AIB was affected in the absence of the other. The effect of ouabain on the transport of taurine, in contrast to that of α -AIB, was however immediate. In view of these observations, the possible relationship between the transport of amino acids and potassium ions was further explored. One approach was to study the effect of the extracellular and intracellular potassium ion on the accumulation of these compounds in the lens. Like the results with α -AIB, absence of potassium ion in the culture medium has no effect on the accumulation of taurine. However, when the potassium content of the lens was first partially depleted by culturing the lens in a potassium-free medium for 22 hours, taurine uptake was reduced by approximately 50% in a potassium-free medium whereas the accumulation of α -AIB was unaffected even under these conditions (Table III).

While these results suggest that the accumulation of taurine is in some way dependent on intracellular potassium levels, the possibility that taurine transport occurs in a system where cation transport is functioning cannot be excluded. It will be recalled (previous AEC reports) that when the ciliary epithelial cells are depleted of intracellular potassium, their capacity to accumulate taurine is nearly abolished in a medium devoid of extracellular potassium. However, the cells depleted of intracellular potassium accumulate taurine when they are incubated in a potassium-containing medium. Moreover, accumulation of amino acid was directly related to the concentration of exter-

nal potassium in the range of 0 - 4 mM concentration which suggests that taurine transport occurs in a system where cation transport is functioning. The rapid turnover of potassium in the lens (4.5 - 6%/hr) could possibly account for the results why the accumulation of taurine in a normal lens is unaffected in a potassium-free medium since the amount of potassium leaking out of the lens into medium during the culture period may be sufficient to activate the pump necessary for the inward movement of this cation. Further studies, however, are needed to substantiate such a hypothesis.

We have also continued our studies on the effect of extracellular sodium on amino acid and inositol transport. It was previously shown that the uptake of amino acids and inositol exhibited a marked sodium dependence. However, the reduced uptake of amino acid cannot be entirely ascribed to the specific effect of sodium ion since it was shown that when rabbit lenses are cultured in a sodium-deficient medium the accumulation of α -AIB, taurine and inositol was dependent on the substances used to iso-osmotically replace sodium. It was further observed that when sodium was replaced by various substances, the lenses became cloudy (except when lithium was substituted for sodium) and the extent of the accumulation of labeled amino acids or inositol was inversely related to the degree of cloudiness. The possibility that the substituents themselves may have some effect on transport mechanisms or that they bring about changes in the physiology of the lens which results in a lower uptake of inositol and amino acids due to changes in the permeability characteristics of the lens membranes was investigated. The lenses were preloaded with labeled inositol for a period of four hours in different media, subsequently transferred to a normal medium (KEI-4) and their rates of efflux measured. Re-entry of the labeled compound into the lens was prevented by including a 10 mM concentration of nonlabeled inositol in the medium.

The results in Fig. 1 show that the rates of efflux from lenses which were incubated in sucrose or choline-containing medium were essentially similar to those of the controls whereas the efflux from lenses cultured in media containing TRIS and TEA⁺ were substantially greater, suggesting a change in permeability characteristics of the lens.

The aforementioned studies clearly indicate the complex nature of the interaction of sodium ions on the transport process of amino acids and further studies are required to establish why sodium ion seems to be an obligatory requirement for the transport of certain amino acids. The present status of the inter-relationship of cations to amino acid transport has been critically reviewed in the accompanying preprint #3.

THE INTERRELATIONSHIP OF AMINO ACID AND CATION DISTRIBUTION IN LENSES OF SPECIES WITH ERYTHROCYTES CONTAINING LOW AND HIGH POTASSIUM

Evidence is accumulating that there are a number of similarities that exist in the biochemistry of the ocular lens and that of the red blood cell. It has been suggested that in both tissues glutathione may play a crucial role. Attention has been drawn to the possibility that similar factors may be involved in the liquefaction of lens fibers in cataracts as well as in red cell hemolysis.

While most mammalian erythrocytes are high in potassium content and low in sodium (like the lens), recent studies have shown that in some domestic sheep erythrocytes the intracellular potassium content is low while the sodium is high. In view of the many striking similarities in the metabolic properties of erythrocytes and the lens it was considered of interest to study the distribution of these ions in the lenses of such animals. If the cation distribution in the lenses is in fact similar to their distribution in erythrocytes, we may inquire as to how the amino acid distribution in this tissue is affected since in most cells the sodium gradient is believed to be the driving force for the transfer of amino acids. In view of these considerations, we have examined the distribution of cations and amino acids in sheep with erythrocytes containing both high and low potassium. Although the results proved to be negative in that the distribution of cations and amino acids in lenses of both HK and LK sheep were similar, these studies demonstrated that free amino acids of sheep lens, like in other species, is higher than in the aqueous humor. Also, the concentration of a number of amino acids in aqueous humor, similar to our earlier findings in rabbits, is higher than in the corresponding plasma, suggesting an active transport mechanism may be involved for the transfer of these compounds across the blood aqueous barrier in these species. The details of this study are summarized in the accompanying preprint ¹.

Fig. 1

Rate of efflux of myoinositol from lenses cultured in media containing various substituents for sodium ion. Lenses were preloaded with 2-³H myoinositol by culturing in Tyrode's medium for a period of 4 hrs. in which sodium ion (137 mEq/l) was iso-osmotically replaced with various substituents. The rates of efflux were determined in KEI-4 medium containing 10 mM nonlabeled inositol. The points are averages for a minimum of 5 experiments. Standard deviations are omitted for the purpose of clarity.

Table II

Effect of various conditions on the accumulation of
C¹⁴-labeled glycyl-glycine in rabbit lens

Time of incubation	Condition of incubation	Concentration M	Conc. of peptide <u>lens water</u> initial medium	% Control
24 hrs	Tracer alone	-	14.3 *	100
	$\bar{C}$ Iodoacetate	10^{-4}	2.6	18
	Glycine	10^{-2}	9.0	63

6 hrs	Tracer alone	-	4.5	100
	Glucose absent		3.8	84

4 hrs	Tracer alone		4.4	100
	Glycyl-glycine nonlabeled	10^{-2}	0.24	5
	Glycine	2×10^{-2}	3.9	88
	L-alanine	5×10^{-3}	3.7	83
	Methionine	5×10^{-3}	4.0	90
	Alanylglycine	5×10^{-3}	0.13	3

* Average of duplicate experiments; all incubations in Tyrode's solution at 37°C.

** Based on measurement of radioactivity present in the lens. Much of the radioactivity is associated with glycine which was formed by the hydrolysis of the peptide after entry into the lens.

Table III

Effect of lack of potassium ion on the accumulation of
C¹⁴-labeled α -AIB and taurine in the lens

Media	Accumulation % of Control $\pm$ S.D. *	
	<u>α-AIB</u>	<u>Taurine</u>
Control (5 mM K ⁺)	100 (4)	100 (6)
No K ⁺	97 $\pm$ 8 (4)	100 $\pm$ 14 (6)
<u>After Preincubation (22 hrs) **</u>		
Control (5 mM K ⁺)	100 (4)	100 (7)
No K ⁺	96 $\pm$ 7 (4)	48 $\pm$ 6 (7)

* Values are expressed as percentage of contralateral controls.

** During the preincubation period the media were replaced at the end of 7 hours and incubation continued for 22 hours. Following preincubation the lenses were cultured for a period of additional 24 hours in fresh media containing either C¹⁴-labeled α -AIB or taurine.

Progress Report

PUBLICATIONS

Feb. 1, 1972 - Jan. 31, 1973

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Phospholipid composition of aqueous humor, plasma and lens in normal and alloxan diabetic rabbits.
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2. Kinsey, V. Everett.
Studies on the crystalline lens XIX. Quantitative aspects of active and passive transport of sodium.
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3. Reddy, V. N.
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To appear in Experimental Eye Research.
4. Reddy, V. N.
Transport of organic molecules in the lens.
To appear in Experimental Eye Research.