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AMINO ACID AND ION TRANSPORT IN THE TOAD CORNEA

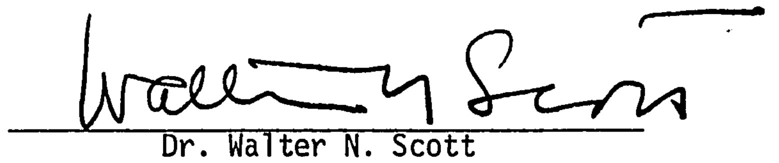
by

Deborra Friedenthal Cooperstein

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1974

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.



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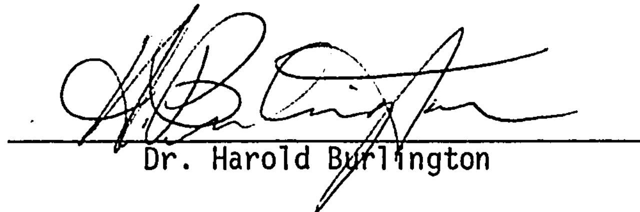

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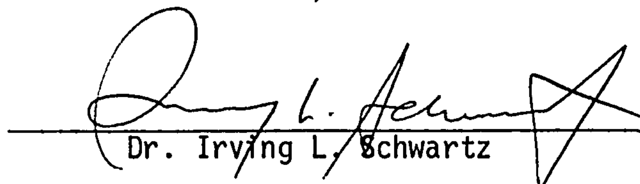

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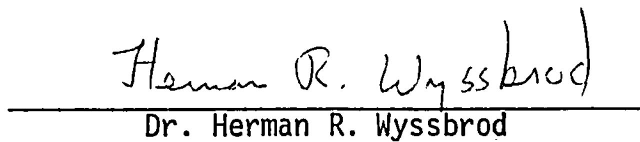

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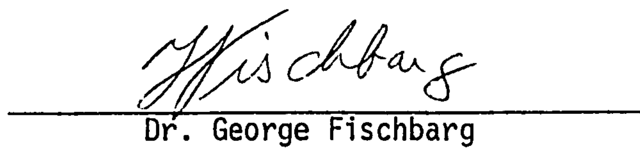

Dr. George Fischbarg

Abstract

AMINO ACID AND ION TRANSPORT
IN THE TOAD CORNEA

by

Deborra Friedenthal Cooperstein

Adviser: Dr. Walter N. Scott

The cornea of the toad, Bufo marinus, is an avascular tissue bounded externally by a tear film (epithelium) and internally by the aqueous humor (endothelium). The cornea has an active protein synthesis apparatus and therefore requires a ready supply of amino acids. α-Aminoisobutyric acid (AIB), a synthetic nonmetabolizable neutral amino acid, enters the toad cornea from the aqueous humor across the endothelium. The accumulation of this amino acid by the toad cornea is mediated by at least three separate transport systems. One system is dependent upon extracellular sodium and actively transports alanine. This system is dependent upon the energy derived from glycolysis for maximal amino acid accumulation and accounts for approximately 60% of the AIB uptake. The second system actively transports leucine and is independent of the concentration of extracellular sodium. Transport via this system is stimulated by both

ascorbic acid and lactic acid. Both these compounds are reducing agents which are found in high concentrations in the aqueous humor. Evidence has been developed that these compounds stimulate amino acid accumulation by donating electrons to an extramitochondrial electron transport system. Transport via this system accounts for approximately 30% of the AIB uptake by the toad cornea. The third system preferentially transports serine and alanine but glycine is excluded from transport via this system. This system is sodium independent and accounts for 10-20% of AIB accumulation.

Active ion transport has been related to the maintenance of normal transparency and hydration in the amphibian cornea. The toad cornea actively transports chloride from the aqueous humor to the tears while sodium is transported in the opposite direction. The active transport of chloride accounts for approximately 60% of the short-circuit current (SCC); the active transport of sodium accounts for the remaining 40%. Physiologic concentrations of ascorbic acid cause a three-fold increase in the endothelial to epithelial chloride transport. Ascorbate has only a slight effect upon the epithelial to endothelial sodium flux. Inhibitors of mitochondrial oxidative phosphorylation inhibit ion transport in the absence of ascorbic acid but have no effect on the ascorbate-induced component of ion transport. Ascorbate is a more effective stimulant of

ion transport while lactate is a more effective stimulant of amino acid transport. This indicates that the transport systems for amino acid accumulation differ from those involved in chloride transport. It also indicates that these transport processes are linked to the extramitochondrial electron transport chain at different sites.

Ascorbic acid is actively transported across the ciliary processes at the expense of considerable metabolic energy. Lactate is derived from glycolysis in the corneal epithelium. These reducing agents may supply energy for the maintenance of corneal transparency.

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CHAPTER I

INTRODUCTION & HISTORY

The outermost, protective layer of the globe of the eye is comprised of the cornea and sclera. The cornea is avascular, having contact with blood vessels only at the limbus. Externally the cornea is bathed by a tear film and interally by the aqueous humor. The aqueous humor of both mammals and amphibians is hyperosmotic to serum (1), and is formed both through ultrafiltration of plasma and active secretory processes (2). The stroma accounts for 90% of the thickness and dry weight of the cornea (3) and is composed of collagen fibrils in a mucopolysaccharide matrix. The tear surface of the cornea is a squamous epithelium 5 or 6 cell layers thick comprising 10% of the dry weight of the cornea (3). The turnover rate of these epithelial cells is approximately seven days and replacement of cells occurs by division of the basal layer (4). The endothelial or aqueous surface of the cornea is a single layer of flattened cells. Approximately 77.5% of the wet weight of the cornea is water (1).

The cornea derives the major portion of its nutrient metabolites such as glucose from the aqueous humor (5, 6), across the corneal endothelium. The permeability of the

endothelium to nonelectrolytes is inversely proportional to their molecular weight (7, 8). The glucose consumption of the *in vitro* rabbit cornea is decreased 80-90% by removal of the epithelium (5) indicating that these cells are responsible for the major part of the corneal metabolism. Glucose is metabolized in the epithelium by aerobic glycolysis, anaerobic glycolysis and the pentose phosphate pathway (9). Eighty five percent of the glucose utilized by the cornea is oxidixed to lactate and only 15% is oxidized completely to carbon dioxide and water (10). Cooling of the intact cornea, which slows metabolism, leads to swelling of the cornea. Corneal swelling and loss in transparency can be reversed upon warming the cornea in media containing glucose (11, 12). Therefore, it seems that the maintenance of normal corneal hydration and transparency depends upon metabolic processes.

Many attempts have been made to determine the relationship between metabolism and corneal hydration, Metabolically driven ion pumps operating in the cornea have been related to the maintenance of normal corneal hydration and transparency in both amphibians (24) and mammals (15). A potential difference exists across the *in vitro* rabbit cornea with the tear surface negative with respect to the aqueous humor (13). A considerable body of evidence indicates the sodium pump is located in the epithelium (14). At this time there is insufficient evidence to implicate

this inward directed ion movement in the maintenance of normal corneal hydration and transparency. Green (15, 16) has postulated that this epithelial sodium pump may serve to control the ionic environment of the stromal mucopolysaccharides and in turn modify the swelling properties of this connective tissue layer. Damage to the endothelium of the rabbit cornea has been found to cause little or no change in the total corneal potential and only a very small potential difference of 0.5 m volts has been measured across the endothelium (17). Nevertheless, Maurice has postulated that the active removal of water occurs across the endothelium and has demonstrated that the isolated endothelium of the rabbit cornea is capable of pumping fluid across its surface against a head of pressure (18). Riley (19) has concluded from his studies that the epithelium behaves as a semipermeable membrane which acts as a barrier preventing access of the tear fluid to the stroma. He has found that Ca^{++} is not required in the epithelial bathing solution to prevent corneal swelling, while it is required in the endothelial bathing solution (20). Riley has also found that only those substances that cause disintegration of the epithelial cell layer promote corneal swelling. He concludes, in agreement with Maurice, that the site of active metabolic control of corneal hydration is in the endothelium. These conclusions are supported by the demonstration of a Na^{+}, K^{+}-dependent ATPase in the endothelium (21).

The cornea of the bullfrog, Rana catesbeiana, actively transports chloride from the aqueous to the tear side (22). This chloride transport accounts for about 80-90% of the short-circuit current. The remaining 10-20% of the short-circuit current is due to an active transport of Na^+ from the tears to the aqueous (23). Sodium is required in the endothelial bathing solution to maintain the chloride transport (24). Ouabain inhibits the short-circuit current and has been found to inhibit both sodium and chloride transport (25). Removal of chloride from the bathing solution leaves a residual Na^+ transport that is not inhibited by ouabain (26). Several authors have developed evidence that both the sodium and chloride pumps are located in the epithelial cell layer because removal of this layer abolishes the short-circuit current and potential difference while removal of the endothelium has no such effect (25, 27, 28). The chloride pump has been implicated in the maintenance of transparency and the prevention of corneal swelling in the frog (24). The ion transport properties of the toad cornea have not been studied.

The aqueous humor is formed by ultrafiltration of plasma and active secretion across the epithelium of the ciliary processes. Ascorbic acid, but not dehydroascorbic acid, is actively secreted into the aqueous humor by the ciliary processes (29). The concentration of ascorbic acid in the aqueous humor is significantly higher than in the

plasma in most vertebrates (30). In fact, the concentrations of ascorbate in the aqueous may approach 20-fold plasma levels (31). In rabbits, guinea pigs, and human beings it has been shown that a rise in the concentration of ascorbic acid in the plasma leads to an increase in the ascorbate concentration of the aqueous humor to a maximal level (32). The concentration of ascorbate in the aqueous was always greater than the concentration in the plasma. Kinsey found that in guinea pigs and rabbits D-isoascorbic acid (an epimer of ascorbic acid) enters the aqueous humor but D-gluco-ascorbic acid does not penetrate the blood aqueous barrier (29). He has also shown that ascorbic acid enters the aqueous humor as a result of an active secretory process in adult animals, while in young animals the predominant factor in determining the distribution ratio is diffusion. Barany and Langham (32) developed evidence that dehydroascorbic acid is not accumulated by the rabbit eye. In their studies of the guinea pig, Linner and Nordstrom (33) found that the uptake of ascorbic acid and D-isoascorbic acid both exhibit saturation kinetics but a much higher plasma level of D-isoascorbic acid is required for saturation. Kinetic analysis of the data showed that the Vmax value for D-isoascorbic acid and ascorbic acid are equal while the Km values for D-isoascorbic acid are four to five fold greater than those for ascorbic acid.

The aqueous humor also contains significant concen-

trations of lactate (31). The high lactate levels originate from the cornea and removal of the corneal epithelium causes a 80-90% reduction in aqueous lactate levels (5). Removal of the endothelium causes no such reduction.

Both the tears (34) and the aqueous humor (35) contain amino acids in concentrations approximating or surpassing those of the plasma. Amino acid concentrations in the aqueous humor of the rabbit range from 0.005-0.614 mmoles/kg H_2O (35). These observations suggest that amino acids are actively transported across the ciliary epithelial cells. The conclusion that some form of active transport is involved in the entrance of amino acids into the aqueous humor is supported by the results of kinetic experiments in which the transport of α -aminoisobutyric acid (AIB) across the ciliary epithelium was measured in vivo (36). This transport process was shown to be saturable. Reddy has shown that the concentration of every free amino acid but arginine, lysine, ornithine, methionine, and hydroxyproline is higher in the corneal epithelium than in the stroma or aqueous humor (37). Reddy's data indicate that the cells of the corneal epithelium are capable of maintaining concentration gradients with respect to the aqueous humor and the stroma, and that the tissue may be capable of transporting amino acids against these gradients. Thoft and Friend (38) have studied the accumulation of AIB by the in vivo rabbit cornea. Their results indicate that AIB enters

across the endothelial surface, the epithelial surface being relatively impermeable to this amino acid. Their study also indicated that the rabbit corneal epithelium is capable of accumulating amino acids against concentration gradients.

The transport of amino acids, like that of a number of other compounds, is generally thought to be a multistep process whereby the amino acid is first bound to an active site on the cell membrane. This active site is considered to be on a membrane-bound protein. This carrier protein-amino acid complex is next translocated across the cell membrane and the amino acid is released into the interior of the cell (39, 40). The carrier protein does not necessarily shuttle back and forth across the cell membrane; the protein may simply catalyze a directional reaction, whereby the amino acid initially present extracellularly is moved to an intracellular location. For example, the carrier protein may have a binding site for substrate that exists in inward-facing and outward-facing conformations, the transport step being the exchange between these conformations.

Amino acid transport has been studied in a variety of tissues. Table 1 summarizes amino acid transport systems elucidated in various tissues. Four separate systems have been implicated in the transport of neutral amino acids. These are: the alanine preferring (A system), the leucine preferring (L system), the ASC system (alanine, cysteine,

Table 1: Vertebrate Amino Acid Transport Systems.

Summary of amino acid transport systems elucidated in some vertebrate tissues. Unnatural amino acids are indicated by parentheses.

Sodium Dependent		Sodium Independent	
ASC System	A System	L System	L^{+} System
Alanine	Alanine	Leucine	Lysine
Serine	Glycine	Isoleucine	Arginine
Cysteine	Methionine	Valine	Ornithine
Proline	Proline	Phenylalanine	Cystine
	Serine	(BCH) *	
	Norleucine	(ACPCA) **	
	(AIB)		
	(MeAIB)		

* BCH = 2-aminobicyclo [2.2.1] hexane-2-carboxylic acid

** ACPCA = aminocyclopentanecarboxylic acid

serine-preferring system), and the glycine system. The A system, as formally defined for the Ehrlich cell, is dependent upon sodium in the bathing medium for maximal amino acid transport (41, 42). It has been found that one sodium ion is translocated inward along with the amino acid molecule (43). Typical amino acids transported by this system are glycine, proline, alanine, sarcosine, serine, methionine and norleucine. AIB and N-methyl-aminoisobutyric acid (MeAIB) are used to characterize this system because it was reported that these amino acids are transported only via the A system. The A system is not present in erythrocytes. Transport via the leucine preferring (L system) is independent of extracellular sodium (44). Reactivity of carrier substrate is proportional to the hydrocarbon mass of the side chain, and thus AIB has a low affinity for this system. The L system is primarily one of exchange and does not lead to concentration gradients. The ASC system is similar to the A system with respect to its rate and its dependence upon the presence of sodium (45). It has a high degree of sterospecificity, transporting only three- and four-carbon aliphatic and hydroxyaliphatic amino acids. MeAIB does not inhibit the transport of natural amino acids by this system indicating that N-methylated amino acids are not transported. This property is helpful in discriminating between the A and ASC systems in a given tissue. The glycine system, which has been extensively studied in avian erythrocytes,

also depends upon extracellular sodium for maximal accumulation of amino acid (46-49). It has been found in the pigeon red blood cell that two sodium ions are translocated with each amino acid molecule. Kinetic studies indicate that both sodium ions must be bound to the carrier before the amino acid can be bound. Only N-methylglycine and N-ethylglycine competitively inhibit glycine uptake. Therefore, it appears that these are the only amino acids that are transported by this system. The glycine system is not present in the Ehrlich cell (43).

Sodium-dependent amino acid transport systems are characterized by a dependence upon extracellular sodium to accumulate amino acids against concentration gradients (41). For example, in the pigeon red blood cell sodium dependent transport systems only obtain a tissue-to-medium distribution ratio of one in the absence of sodium (51). In the presence of sodium, tissue amino acid concentrations may be as great as ten-fold the concentration of the amino acid in the medium. The normal gradient of sodium is such that the extracellular concentration of sodium is much greater than the intracellular concentration. The sodium gradient hypothesis suggests that the energy for amino acid accumulation is derived from the electrochemical potential gradient of sodium across the cell membrane. It is proposed that sodium and the amino acid both bind to the carrier protein; the binding of sodium inducing a conformational

change in the carrier protein which permits either binding of the amino acid, migration of the carrier protein-amino acid complex, or both. The transport of the amino acid across the cell membrane cannot be classified as "primarily active" because the energy for the translocation process is derived from the electrochemical gradient of sodium across the cell membrane. In fact, the maintenance of low intracellular concentrations of sodium is the only step that requires the direct input of metabolic energy and is, therefore, the active transport step in this process.

Evidence for this hypothesis is provided by the experiments of Vidaver who used lysed and restored pigeon red blood cells (47). He found that if intracellular sodium concentrations were maintained at a high level while extracellular sodium concentrations were low, glycine was pumped out of the cell as efficiently as it was pumped into the cell with a similar sodium gradient in the normal direction. No net flux of amino acid was observed in the absence of a sodium gradient. There was no evidence that the potassium gradient has any effect upon the distribution of glycine. Eddy has demonstrated that the sodium electrochemical potential is the major determinant of glycine accumulation in cyanide-poisoned ascites tumor cells, and that there is a slight additional contribution from the potassium gradient (51). Eddy's studies did not exclude the possibility that additional energy from cellular metabolism is involved in

normal amino acid accumulation. Schafer and Heinz examined AIB transport in Ehrlich ascites tumor cells and found that the net AIB flux was reversed only after very drastic inversion of the sodium and potassium electrochemical potential gradients (52). They concluded that another driving force must be present in addition to the electrolyte gradients. It would appear that the energy for the accumulation of AIB in these cells is derived from two sources, namely cotransport with sodium and metabolic energy derived directly from cellular metabolism. Johnstone has shown that in Ehrlich ascites cells in which the sodium and potassium gradients have been abolished, but cellular ATP is available, glycine accumulation is not diminished (53). In most studies in which sodium and potassium gradients are reversed the extracellular sodium concentration is decreased and Johnstone proposes that it is this decrease in the extracellular sodium concentration rather than the reversal of the sodium or potassium gradient across the cell that leads to a decrease in amino acid accumulation.

It is considered that sodium may be necessary for the binding of the amino acid to the carrier or for the movement of the carrier-amino acid complex across the cell membrane. The former would be revealed by a sodium effect on the K_m for amino acid transport, the latter by a sodium effect on V_{max} (54). For glycine entry into pigeon red blood cells Vidaver has found that the K_m term is governed

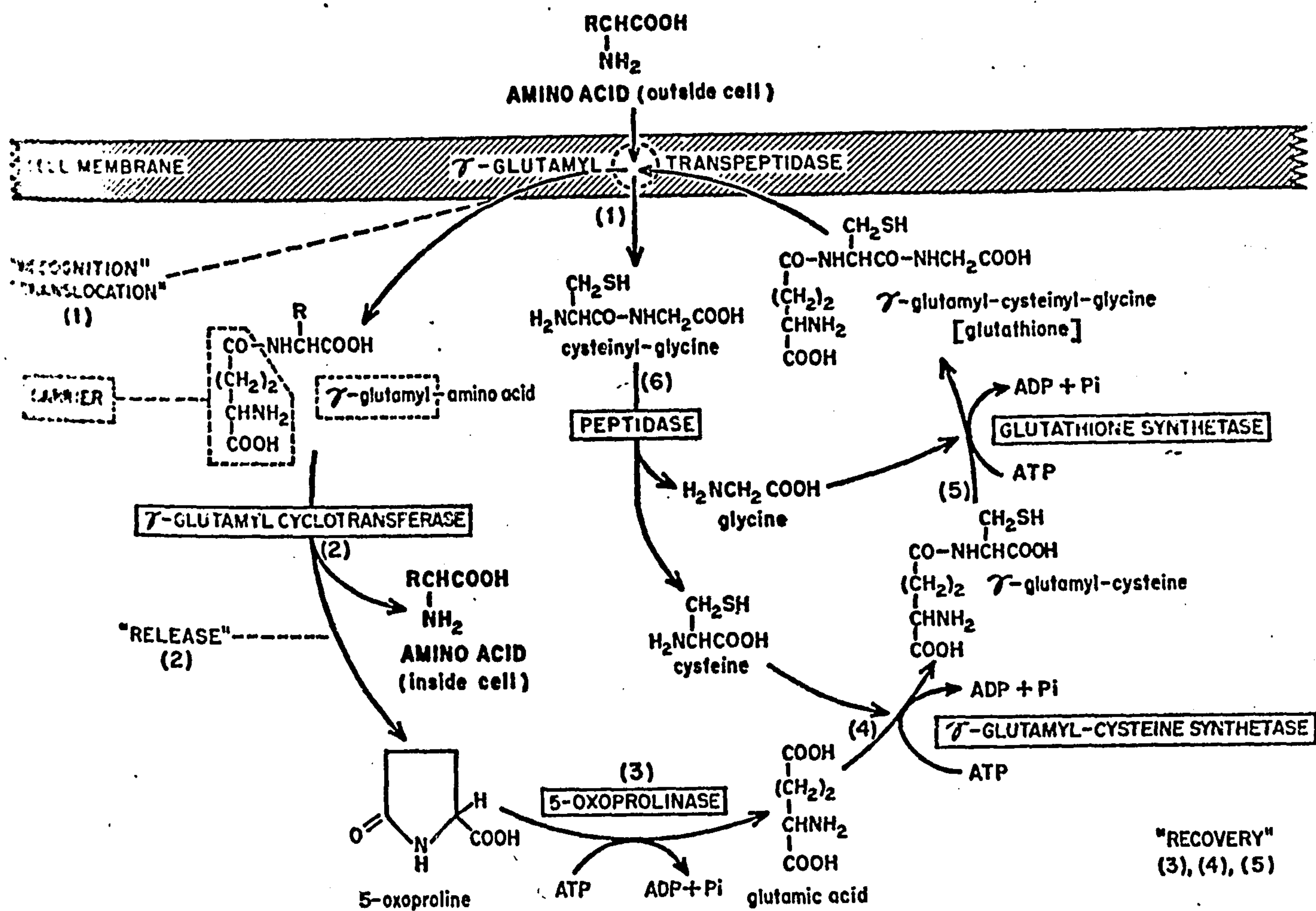
by sodium (46). For glycine entry into ascites cells it has been shown that the V_{max} term is decreased when the external sodium concentration is decreased while the external potassium concentration is held constant (51, 55). The K_m term was not effected by removal of sodium. Jacquez, in his studies of alanine and AIB accumulation by the Ehrlich cell, showed that the K_m for uptake of both amino acids was increased while V_{max} was decreased when choline was substituted for sodium (56). Thomas and Christensen have studied the nature of sodium and amino acid cotransport in the ASC system of the Ehrlich cell, rabbit reticulocyte and pigeon erythrocyte (57). They suggest that the two cosubstrates bind in juxtaposition at the receptor site, and that a substantial portion of the cotransport interaction occurs directly between the two substrates, the amino acid and sodium. They further conclude that translocation is not the rate-limiting step in amino acid accumulation and that the order in which the two substrates bind to the carrier is unimportant.

Specific transport systems for the accumulation of dicarboxylic amino acids and cationic amino acids have also been described in the Ehrlich cell (58, 59), kidney (60, 61) and other tissues. It has been shown that sodium or lithium and a neutral amino acid can act together to competitively inhibit the transport system for cationic amino acids (59).

Reabsorption of amino acids in the proximal tubule of the kidney occurs via transport systems similar to those previously described. Recently, Meister has proposed a mechanism for amino acid transport in the mammalian kidney and brain (62). The proposed mechanism is illustrated in Figure 1 and his basic proposition is that glutathione acts as a donor of the γ-glutamyl group which serves as a carrier in amino acid transport. Unfortunately, this hypothesis does not account for the sodium dependence of the accumulation of certain amino acids, nor does it explain the specificity of the various amino acid transport systems. Only if one postulates the presence of isozymes of γ-glutamyl transpeptidase or the existence of separate binding proteins can one accept this hypothesis to explain the mechanism for all amino acid transport processes.

Figure 1: The γ-glutamyl cycle

The proposed γ-glutamyl cycle for amino acid transport across cell membranes (62).



CHAPTER II

M A T E R I A L S & M E T H O D S

A. Amino Acid Accumulation Experiments

The entire cornea with a ring of sclera was excised from the globe of Bufo marinus toads of Colombian origin (Tarpon Zoo, Tarpon Springs, Florida). The corneas were incubated at room temperature (about 25°C) in 5 ml. of a modified Conway-Ringer's solution consisting of 83.5 mM NaCl, 17.7 mM $NaHCO_3$, 4 mM KCl, 0.8 mM $MgSO_4$, 0.8 mM KH_2PO_4, 11 mM glucose, and 1.5 mM $CaCl_2$. Bubbling with a gas mixture containing 1% CO_2 established a pH of 8.0 at 25°C. The incubation media also contained 1.0 μCi of the requisite amino acid: ^{14}C-AIB (20 mCi/mmole), L- [^{14}C] -alanine (130 mCi/mmole), or L- [^{14}C] -leucine (45 mCi/mmole), 5,0 μCi of[^{3}H] -inulin (4.21 Ci/gm) or [^{3}H] -mannitol (2.65 mCi/mmole) and unlabeled amino acids in concentrations ranging from 10 μM to 10 mM. The radioactively labeled compounds were purchased from New England Nuclear, Boston, and ICN, Cleveland, and the purity of all amino acids were verified by thin layer chromatography in acetic acid-butanol-water, 20:80:20 (v/v). In the studies of the pH dependence of amino acid accumulation the "Good" buffers (63)

were substituted for the bicarbonate in our Conway-Ringer's to obtain a trajectory of pH's: 17.7 mM MES [2-(N-morpholine) ethane-sulfonic acid] (pH 6.8 to 7.0), TES [N-Tris-(hydroxymethyl) methyl-2-aminoethane sulfonic acid] (pH 7.0 to 8.0), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.0 to 8.0) or BICINE [N,N'-bis (2-hydroxyethyl) glycine] (pH 8.0 to 9.0).

At the end of the incubation period, the tissue was removed from the medium and rinsed with 2 ml. of Ringer's solution. A piece of cornea 6 mm. in diameter was excised from the center of the tissue with a Paton trephine, and dissolved in 2 ml. of Soluene-100, a quaternary amine (Packard Instrument Co.). One hundred microliter samples of the incubation media were also dissolved in 2 ml. of Soluene-100 for counting. Fifteen ml. of a toluene-base scintillant (composition: 4 g. PPO, 50 mg. POPOP per liter of toluene) were added to all samples and the samples counted using two channels of a Packard Model 3320 Tri-Carb liquid scintillation spectrometer. The channels were set so that ^{3}H was completely excluded from the ^{14}C channel (less than 0.01% spillover of ^{3}H into the ^{14}C channel).

The ^{14}C spillover into the ^{3}H channel was kept at a low level, approximately 10%. Quenched standards (Nuclear Chicago) were counted at the end of each experiment and the channels' ratio and corresponding efficiencies were stored in a computer program on a Honeywell

1648 computer. The percentage spillover of ^{14}C into the ^{3}H channel was also determined. The counting data was retrieved on paper tape for transmittal to the computer. The program was designed to calculate the channels' ratio for each sample, and, by linear interpolation, to calculate the efficiency at which each sample was counted. Then, using the following equations, the disintegrations per minute (DPM) of each isotope was calculated for each sample.

$$^{14}C \text{ DPM} = \frac{^{14}C \text{ CPM}}{^{14}C \text{ Efficiency}}$$

$$^{3}H \text{ DPM} = \frac{^{3}H \text{ CPM} - {}^{3}H \text{ CPM} \times \text{Fractional Spillover } {}^{14}C \text{ into } {}^{3}H}{^{3}H \text{ Efficiency}}$$

The extracellular space of each tissue was determined from the ^{3}H counts and this value was used to correct for extracellular (^{14}C)-labeled amino acid in each tissue. In every case, one cornea from each toad was treated as an experimental tissue, while the contralateral cornea of the same animal was used as its control.

B. Amino Acid Accumulation Experiments - Epithelium and Endothelium Treated Separately

Corneas with their surrounding sclera were mounted as a membrane between two halves of a Lucite chamber. The cornea was first placed, endothelial side down, on a moist-

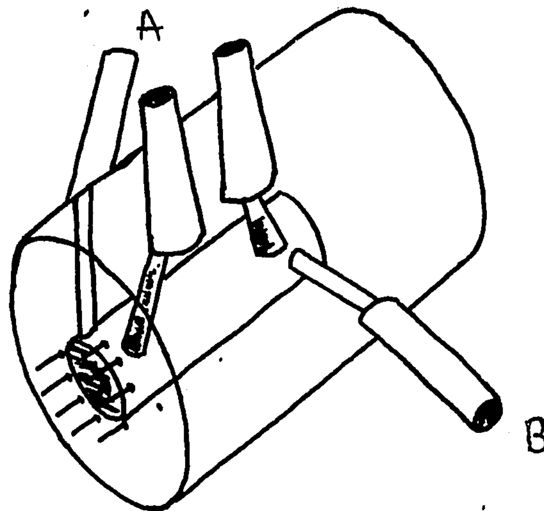
ened Lucite adapter. This adapter was especially equipped with metal guides which allowed the cornea to be placed over the central opening of one half of the chamber and evenly impaled on the aluminum pins located on this chamber half. The adapter was then removed and the matching chamber half clamped in place. The final configuration is two reservoirs of approximately 0.5 ml separated by the sheet of cornea. The surface area of the cornea exposed to the solutions on each side was 0.5 cm^2. Each chamber half was connected by an inlet and outlet to a glass reservoir, giving a total volume of 5 ml. An airlift pump in the outlet tubing served both to circulate the bathing media and to maintain the desired degree of saturation of oxygen and CO_2. The cornea was bathed on each surface with 5 ml of our modified Conway-Ringer's except in those experiments in which HCO_3 was omitted. The natural shape of the cornea was maintained by applying a small ($\sim$1 cm) hydrostatic pressure from the endothelial side. After incubation of the cornea with the requisite amino acids the cornea was carefully removed from the chamber, rinsed, dissolved, and the radioactivity determined exactly as described in Section A.

C. Electrical Measurements

Corneas were mounted as a membrane between two halves of an Ussing-style Lucite chamber (Figure 2) exactly as described in Section B. This chamber differed from that

Figure 2: Cornea Incubation Chambers

This is one-half of the chamber that was used for short-circuited ion transport studies across the toad cornea. The other chamber half is identical except that it contains holes into which the pins fit. The cornea is impaled upon the pins and then the other chamber half is fitted into place. At A polyethylene tubing filled with an agar-sodium Ringer solution was inserted into the chambers to monitor the potential difference across the cornea. At B agar-sodium Ringer filled polyethylene tubing was inserted to monitor the short-circuit current (see Text).



described previously in that each chamber half had two extra tubes in addition to those that served to circulate the Ringer solution. Polyethylene tubing filled with an agar-sodium Ringer solution was placed in tubes A and A'. This agar-filled polyethylene tubing made contact with saturated KCl-calomel electrodes. The calomel cells were connected to the measuring and recording equipment which consisted of Keithley 200B millivoltmeters and Heath EU-20 recorders. This configuration allowed us to monitor the potential difference across the cornea. For measurement of the short-circuit current agar bridges connected the tubes labeled B and B' to plastic beakers of Ringer solution which contained the leads from an automatic voltage-clamp apparatus. The voltage-clamp apparatus sends sufficient current through these electrodes to maintain the potential difference (monitored by the other pair of electrodes) at zero. This current is continuously recorded, except for brief intervals when the spontaneous potential difference is measured, and is equivalent to the amount of ions transported by the tissue. This set-up is similar to that originally described by Ussing and Zerahn (64).

D. Unidirectional Ion Fluxes

Corneas, mounted in the Lucite chambers as described above, were short-circuited and allowed to stabilize for 30-45 minutes. One-tenth milliliter of either

^{36}Cl (4.3 mCi/mg) or ^{22}Na (300 mCi/mg) (Amersham-Searle) was added to the chamber compartment and 2.0 ml samples taken at 20-minute intervals from the opposite compartment. The total volume of this compartment was kept constant at 5 ml by replacement of 2 ml of Ringer's each time a 2 ml sample was taken. 25 µl samples were taken from the side to which the radioactivity was initially added and were diluted to 2.0 ml with Ringer's solution before counting. Samples were dissolved in 15 ml of a liquid scintillation mixture consisting of 33.3% Triton X-100, 66.7% toluene with 4 g. PPO and 50 mg. POPOP per liter. The samples were counted in a Packard 3320 Tri-Carb liquid scintillation spectrometer.

E. Materials

Ouabain, adenosine 3',5' monophosphate (cAMP), N^6,2'-O-dibutyryl-cyclic adenosine monophosphate, L-ascorbic acid, D-isoascorbic acid, leucine and methacholine (Mecholyl) were purchased from Sigma Chemical Co., St. Louis, Missouri. Unlabeled leucine, alanine, glycine, and serine were purchased from Fisher Scientific Co. and α-aminoisobutyric acid (Lot #7503) from K & K Laboratories, Plainview, New York. Phospholine iodide was purchased from Ayerst Laboratories, New York, N.Y. Dehydro-ascorbic acid was prepared immediately before use by a modification of the method of Roe and Keuther (65). A 1% solution of ascorbic acid in

0.1 N acetic acid was prepared. Activated charcoal (0.05 g. per ml) was added and the mixture was shaken vigorously for 20 minutes and then filtered. Conversion of all of the ascorbic acid to dehydroascorbic acid was ascertained by thin layer chromatography.

CHAPTER II

AMINO ACID UPTAKE

A. Extracellular Space Measurements

The cornea is a complex tissue composed of three distinct layers. The stroma accounts for approximately 90% of corneal weight (3) and it is considered an acellular permeable mesh work. Therefore, in order to accurately determine the accumulation of amino acid by the cells in the tissue it was necessary to measure the apparent extracellular space in each toad cornea to correct for the diffusion of amino acid into this acellular stroma.

We determined both the extracellular space and wet weight of the cornea. The corneas were incubated in Ringer's solution containing $[^{3}H]$-inulin and $[^{14}C]$-mannitol or $[^{3}H]$-mannitol and $[^{14}C]$-inulin. At the end of the in cubation period the corneas were removed, rinsed with Ringer's solution, blotted on filter paper, and a 6 mm button excised from the center of the cornea. The button was weighed on a Smith Roller Balance and then dissolved in 2 ml. of Soluene-100 for counting. As shown in Table 2, the wet weight of the cornea increases when the cornea is incubated in Ringer's solution. The extracellular space of the cornea also increased during a four-hour incubation period in Conway-Ringer's solution. When the increase in the wet

Table 2: Measurement of Extracellular Space in Toad Cornea

Corneas were incubated in 5 ml. of Ringer's solution buffered at pH 8.0 with bicarbonate (17.7 mM) and containing $[^3H]$-inulin (5.0 μCi) and $[^{14}C]$-mannitol (2.0 μCi) or ^{14}C-inulin (2.0 μCi) and ^3H-mannitol (5.0 μCi). Following incubation for the indicated period of time a 6 mm. button of tissue was excised, blotted briefly on Whatman filter paper, and weighed on a Smith Roller Balance. The excised button was then dissolved in Soluene-100 and the radioactivity was measured in the dissolved tissue and in the incubation medium. The extracellular "space" of the corneal fragment was calculated from these values. The results are expressed as the mean ± the Standard Error of the Mean (SEM). The number of experiments are shown in parentheses.

Incubation time	Inulin Space (ul)	Mannitol Space (ul)	Wet Weight (mgm)
0			5.6 ± 0.3 (6)
15 min.	1.68 ± 0.31	2.67 ± 0.35	8.53 ± 0.58 (12)
1 h.	2.97 ± 0.22	3.70 ± 0.19	10.57 ± 0.35 (12)
4 h.	5.16 ± 0.37	5.55 ± 0.42	14.23 ± 1.34 (12)

weight of the corneas was compared with the increase in the extracellular space, it was found that the extracellular space, as measured with mannitol, increased at approximately the same rate as the wet weight. From 15 minutes to 1 hour of incubation the mannitol space increased 28% while the wet weight increased 19%. During the same time period the extracellular space, as measured with inulin, increased much more (43%) than the wet weight. During the remaining 3 hours of incubation, the inulin space increased 42% while the mannitol space increased 33% and the wet weight increased 29%. These results indicate that mannitol is probably a better indicator of corneal extracellular space than inulin.

As shown in Table 3, the extracellular space of the cornea increases significantly when incubated in a Ringer solution in which sodium has been replaced by choline. This increase in extracellular space is dependent upon the length of time the cornea is preincubated in choline Ringer. After a preincubation period of one-half hour there was no significant change in the extracellular space. When the preincubation period in choline Ringer was increased to 2.5 hours the extracellular space, as determined by either mannitol or inulin, was increased significantly over control corneas incubated in Ringer's solution. These results suggest that extracellular sodium is important for the maintenance of normal corneal hydration.

Table 3: Sodium Dependence of Corneal Hydration

One cornea from each toad was first incubated for the indicated period in a Conway-Ringer in which sodium was replaced by choline (100 mM). The sodium concentration in the choline Ringer was measured using a pHM 52 electrometer and sodium electrode and was found to be 10 µM. After the initial incubation period, ^{3}H-inulin (5.0 µCi) or ^{3}H-mannitol (5 µCi) was added to the media. The corneas were incubated for an additional 60 minutes and the amount of radioactivity in the tissue and medium was determined. The paired control corneas were handled identically except that the Ringer solution contained sodium (100 mM). The results are expressed as the Mean ± SEM. The number of experiments is shown in parentheses. Paired data was subject to Student's t Test: $p < 0.02$ is indicated by ǂ, and $P < 0.001$, by §.

Extracellular Space (ul)		Preincubation Time (hr)	% Change in Extracellular Space
Conway Ringers	Choline Ringers		
Inulin			
3.49 ± 0.24(13)	2.93 ± 0.21(13)	0.5	-14.8 ± 9.2(13)
2.95 ± 0.18(13)	4.16 ± 0.46(13)	0.2	47.5 ± 17.0(13)ǂ
Mannitol			
4.23 ± 0.28(9)	5.98 ± 0.40(9)	2.5	44.2 ± 9.9(13)§

B. Amino Acid Accumulation Studies

α-Aminoisobutyric acid (AIB) was chosen for our initial studies on amino acid accumulation by the cornea. AIB is a non-metabolizable neutral amino acid. The cells of the corneal epithelium exhibit a rapid turnover rate and therefore they must have an active protein synthesis apparatus. The use of natural amino acids in the presence of protein synthesis inhibitors was avoided because these inhibitors might have undesirable effects upon transport.

In our initial studies toad corneas were mounted in Lucite chambers so that each surface of the cornea could be treated separately and we could thereby determine across which surface amino acid accumulation occurred. When $[^{14}C]$ -AIB and $[^{3}H]$ -inulin or mannitol were added to the epithelial bathing solution of one cornea and to the endothelial bathing solution of the contralateral cornea, it was found that 96% of the AIB accumulated by the cornea entered across the endothelial surface of the cornea. When the degree of inhibition of AIB uptake across the endothelium in the presence of competing amino acids was compared to the inhibition when both surfaces of the cornea were exposed to AIB, we found identical results. The effects of iodoacetate upon AIB entry through the endothelium were identical to the effects observed when both surfaces were exposed to AIB. The small amount of AIB entering across the epithelium was not altered by metabolic inhibitors or high concentra-

tions of unlabeled competing amino acids. These results indicated that AIB was entering the cornea only across the endothelial surface but gave no indication of either the site of active transport or accumulation.

Once we determined that amino acid entry was occurring across only one surface of the cornea, we were able to immerse the entire cornea in the incubation medium, thereby obviating the time-consuming task of mounting each individual tissue in a Lucite chamber. This significantly simplified the experimental procedure.

AIB is progressively accumulated by the toad cornea *in vitro* (Figure 3). Using the mannitol extracellular space value to correct for extracellular $[^{14}C]$-AIB in each tissue, we found that the uptake of this amino acid is linear for a period of at least four hours. When inulin is used to correct for the extracellular $[^{14}C]$-AIB in each tissue, the uptake is linear from one to four hours. As shown in Figure 4 there is a break in the AIB uptake curve at approximately one hour. Inulin is a large (m.w. = 5000) polysaccharide polymer and this non-linear uptake curve is probably caused by the slow diffusion of inulin into this complex tissue. Mannitol (m.w. = 181) presumably equilibrates between the tissue and the medium much more rapidly than inulin. Mannitol is preferable on theoretical grounds for amino acid uptake studies because the molecular weight of this saccharide approximates that of the amino acids studied.

Figure 3: Corneas were incubated with 1 μCi (0.5 mM) [^{14}C]-AIB and 5 μCi [^{3}H]-mannitol. At the end of the incubation period the amount of radioactivity in a 6 mm button excised from the center of the cornea was determined. The results are expressed as the Mean ± SEM. The data represent an average of six experiments.

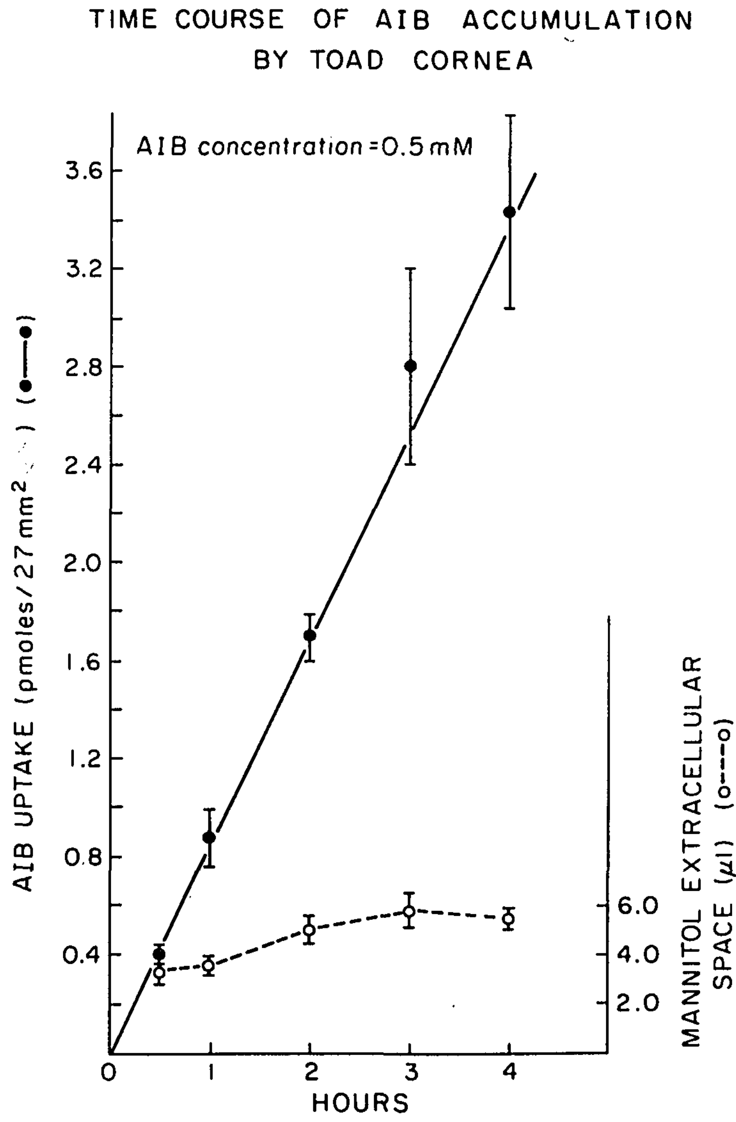
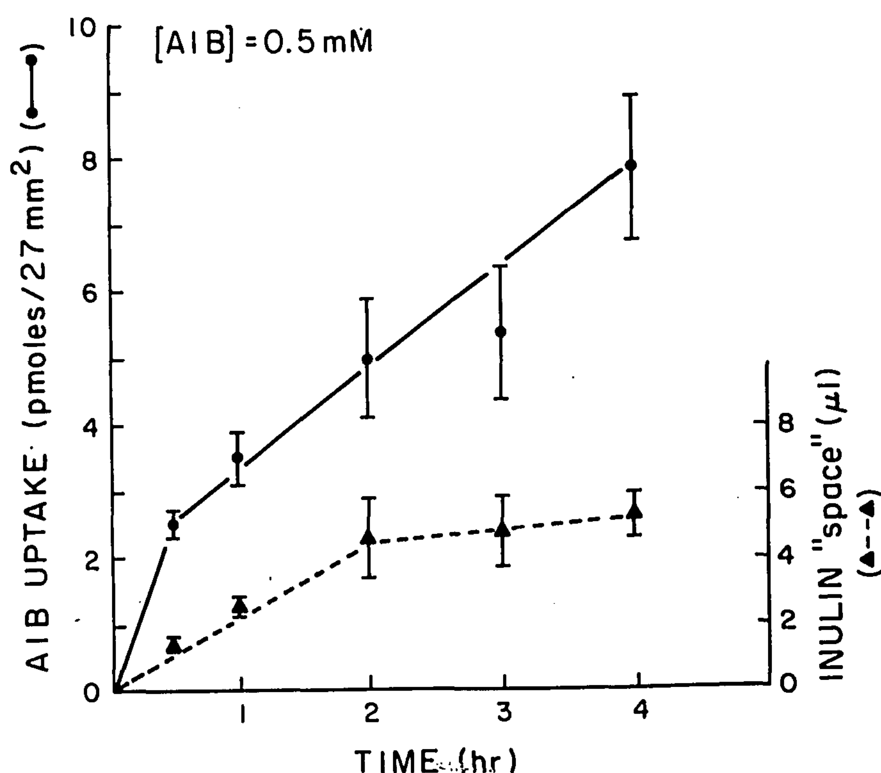


Figure 4: Corneas were incubated in 5 ml. of modified Conway-Ringer containing 1 μCi (0.5 mM) [^{3}H]-inulin. At the end of the incubation period, corneas were removed and rinsed with 2 ml. of Ringer's solution. A 6 mm button was excised from the center of the cornea and dissolved in 2 ml. of Soluene-100. The radioactivity in each sample was determined. The results are expressed as the Mean ± SEM. The data represent an average of six experiments.



To determine the optimum pH for AIB accumulation, we replaced the $NaHCO_3$ in the modified Conway-Ringer by an equal concentration of TES, BICINE, MES, or HEPES, titrated the solution to the desired pH, and bubbled the medium with 100% oxygen. Replacement of bicarbonate by these "Good" buffers had no apparent effect upon amino acid accumulation by the cornea. The uptake of AIB at pH 8.0 was 0.05 ± 0.01 (Mean $\pm$ SEM) pmoles/mm^2/hr in medium buffered with bicarbonate and 0.05 ± 0.01 (Mean $\pm$ SEM) pmoles/mm^2/hr in Ringer's solution buffered at pH 8.0 with TES. The optimum pH for accumulation of this amino acid by the toad cornea is approximately 8.0 (Figure 5). When leucine uptake was subsequently studied in the same manner, it was found that the uptake curve showed a broad pH optimum ranging from pH 7.5 to pH 8.5 (Figure 6). Substitution of the "Good" buffers for bicarbonate did not affect leucine accumulation by the cornea.

As shown in Figure 7, the accumulation of AIB by toad cornea exhibits a concentration-dependent uptake of amino acid indicating the presence of carrier-mediated transport. Saturation of AIB uptake in the cornea was achieved at a concentration of approximately 5 mM. The data were plotted by both the Michaelis-Menten ($^1/v$ vs. $^1/[S]$) and as $^S/v$ vs. $[S]$. Neither treatment of the data yielded a linear plot, suggesting that more than one mechanism is involved in the accumulation of AIB by the

Figure 5: The "Good" buffers were substituted for bicarbonate in modified Conway-Ringer's to obtain a trajectory of pH's: 17.7 mM MES (pH 6.8 to 7.0), TES (pH 7.0 to 8.5), HEPES (pH 7.0 to 8.0) or BICINE (pH 8.0 to 9.0). The incubation media contained 1 μCi of $[^{14}C]$-AIB (5.0 μM) and 5.0 μCi of $[^{3}H]$-inulin and was bubbled with 100% O_2. Tissues were incubated for one hour. The results are expressed as the Mean ± SEM. The data represent an average of five experiments.

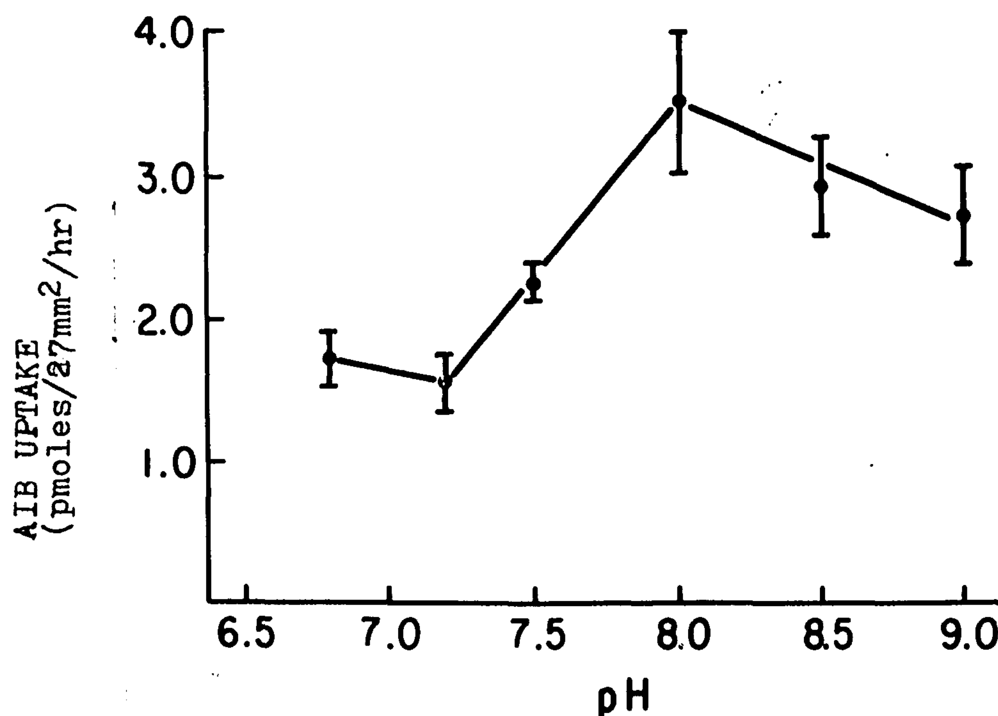


Figure 6: "Good" buffers were used to obtain a trajectory of pH's in the modified Conway-Ringer as described in Materials and Methods. The incubation media contained 1 μCi (1 mM) of [^{14}C]-leucine and 5 μCi of [^{3}H]-inulin and was bubbled with 100% O_2. The results are expressed as the Mean ± SEM. The data represent an average of six experiments.

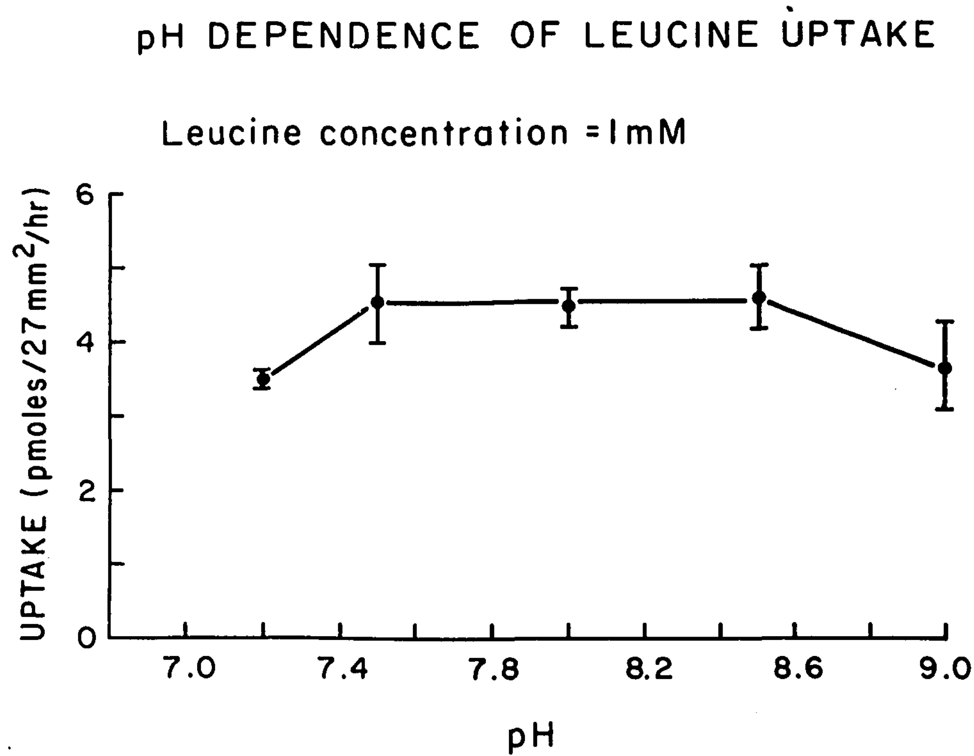
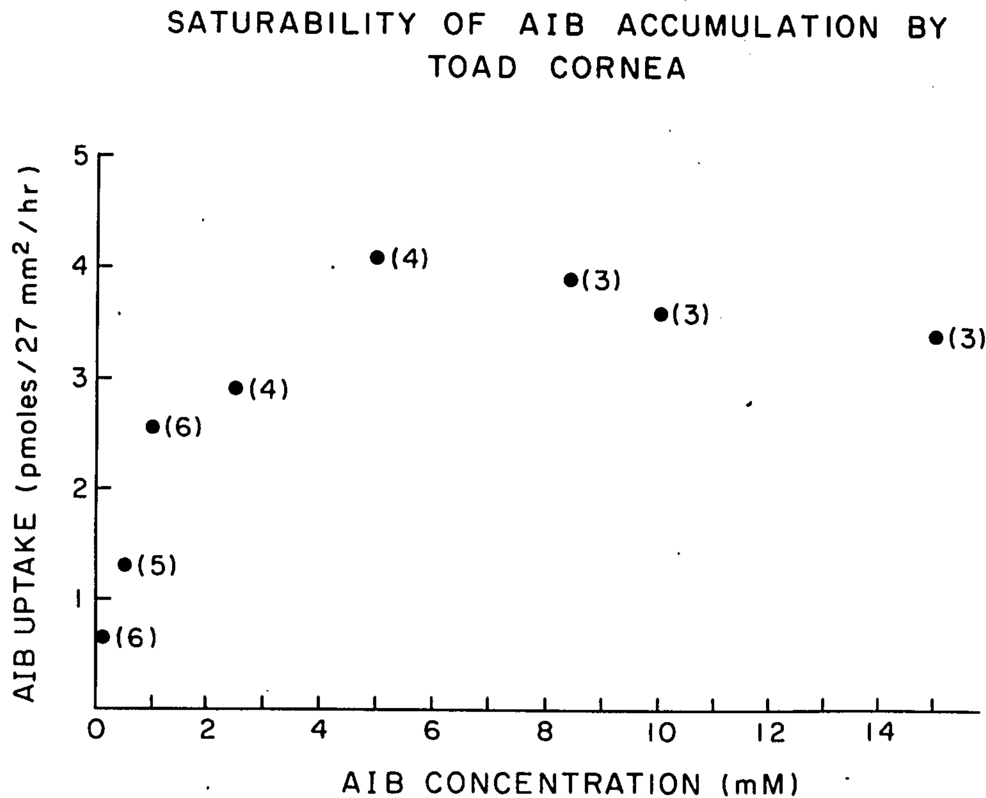


Figure 7: Corneas were incubated in modified Conway-Ringer's, pH 8.0 with 1 µCi [^{14}C]-AIB, 5 µCi [^{3}H]-mannitol and cold AIB; the AIB concentration ranged from 0.1 mM to 15.0 mM. After one hour of incubation the corneas were rinsed with Ringer's solution and a 6 mm button was excised from the center of the cornea with a Paton trephine. The weight of the excised tissue was 10.6 ± 0.4 (SEM) mgm (12) and the mannitol extracellular space was 3.70 ± 0.19 µl (12). The number of experiments is shown in parentheses.



toad cornea.

In order to ascertain the substrate specificity of the transport system(s) responsible for the accumulation of AIB we measured the effects of a series of neutral amino acids upon the uptake of AIB. Although it had been shown in a number of tissues that AIB was transported primarily by the A system our saturation studies with AIB indicated that this amino acid is transported by more than one system in the cornea. Table 4 shows that leucine, glycine and alanine each significantly inhibited the uptake of AIB. If AIB were transported only by the A system, leucine should have had no effect upon AIB uptake. Moreover, the degree of inhibition of AIB uptake was related to the concentration of the competing natural amino acids. The maximal inhibitory effect of those natural amino acids studied was achieved at a concentration of 2.5-5.0 mM. The natural amino acids have no inhibitory effects on each other's uptake.

Table 4 also shows that the effects of the various natural amino acids are additive. The uptake of AIB was reduced 18.3% by the addition of 10 mM leucine, and 57.1% by the addition of 10 mM alanine to the incubation media. The uptake of AIB was reduced even further to 79.3% when 10 mM leucine plus 10 mM alanine were added to the incubation media. The effects of these two amino acids are evidently additive indicating further that more than one transport system is involved in AIB uptake. The effects

Table 4: Competition of Natural Amino Acids for the Uptake of AIB by the Cornea.

Corneas were incubated for one hour at room temperature in modified Conway-Ringer, pH 8.0, containing 0.5 mM AIB and unlabeled amino acids. In the studies in the upper portion of the Table, the concentration of a single amino acid was varied from 0.1 to 10 mM. In the lower portion, the effects of 2 or 3 amino acids, each at a concentration of 10 mM, were studied. Paired control corneas were treated in the same manner except that the medium contained no competing natural amino acids. AIB uptake was 3.12 ± 0.12 pmoles/27 mm^2/hr in 104 control experiments. Inhibition of $[^{14}C]$-AIB uptake is expressed as the Mean ± SEM relative to the paired control corneas. The number of experiments is shown in parentheses.

Concentration of Competing Amino Acids (mM)	% Inhibition of 3-Aminoisobutyric Acid Uptake by Natural Amino Acids		
	Alanine	Glycine	Leucine
0.1	1.4 ± 8.0 (7)	3.0 ± 10.6 (8)	10.0 ± 16.8 (3)
0.5	17.6 ± 5.4 (6)	3.6 ± 10.6 (3)	21.2 ± 10.6 (9)
1.0	23.1 ± 6.8 (3)		21.3 ± 11.8 (6)
2.5	54.3 ± 1.7 (3)	40.1 ± 6.0 (3)	26.8 ± 8.0 (5)
5.0	40.0 ± 6.0 (4)	41.0 ± 7.8 (8)	29.0 ± 10.5 (4)
10.0	57.1 ± 5.6 (9)	36.1 ± 14.1 (3)	18.3 ± 7.9 (5)
	Leucine Glycine	Leucine Glycine Serine	Leucine Alanine
10.0	63.8 ± 2.5 (11)	76.7 ± 1.2 (11)	79.3 ± 7.8 (5)

of 10 mM leucine and 10 mM glycine upon AIB were also additive, inhibiting uptake by 63.8%. The addition of 10 mM serine to the latter incubation media (leucine plus glycine) further reduced AIB uptake to 76.7% of the control value. Thus, AIB uptake in the presence of 10 mM leucine plus 10 mM glycine (1.358 $\pm$ 0.063 pmoles/27 mm^2/hr) was reduced by approximately 35% when 10 mM serine was added to the incubation medium (0.803 $\pm$ 0.051 pmoles/27 mm^2/hr). To further test that the leucine-preferring and alanine-preferring systems were each saturated with their respective substrates, we increased the concentration of the natural amino acids even further. In six pairs of corneas 20 mM leucine plus 10 mM glycine inhibited AIB uptake 59.5 $\pm$ 6.0% (1.659 $\pm$ 0.094 pmoles/27 mm^2/hr). 10 mM leucine plus 20 mM glycine inhibited uptake by 53.6 $\pm$ 4.5% (1.652 $\pm$ 0.213 pmoles/27 mm^2/hr). Therefore, the inhibitory effects of serine upon AIB uptake seem to be the result of a specific inhibition and these data indicate that AIB is also being accumulated by a system that has a high affinity for serine.

We next studied the effects of sodium upon the uptake of AIB by the cornea. If AIB was being transported solely by the A system one would expect that replacement of sodium by choline should significantly reduce the accumulation of AIB by the cornea because the normal sodium gradient across the cells would be removed. One would also expect AIB uptake to be reduced to the same extent as alanine up-

take. As shown in Table 5, replacement of sodium by choline does significantly reduce the accumulation of AIB. The degree of inhibition depended upon the length of time the cornea was preincubated in choline Ringer's solution before the addition of radioactively labeled AIB, but never amounted to more than 50%. When the effects of sodium replacement upon the accumulation of alanine and leucine were studied separately, alanine uptake was inhibited whilst the accumulation of leucine was not affected. The relative effects of the longer preincubation time upon alanine uptake were similar to those observed with AIB, with the inhibitory effects doubling between the 0.5 hr and the 2.5 hr period. Nevertheless, the inhibitory effects of removal of sodium upon alanine uptake were always 50-75% greater than on AIB uptake. We also found that the small residual uptake of AIB in the presence of saturating concentrations of both leucine and glycine (0.33 $\pm$ 0.007 (SEM) pmoles/mm^2/hr) (Table 3) was not reduced further when sodium was replaced by choline (0.044 $\pm$ 0.003 (SEM) pmoles/mm^2/hr). This suggests that the small residual uptake of AIB seen under these conditions is mediated by a system not related to external sodium concentrations and thus seems to rule out the ASC system, which is sodium-dependent.

In order to determine the sources of energy for the accumulation of AIB we studied the effects of anaerobiosis and certain metabolic inhibitors upon AIB uptake. Neither

Table 5: Sodium Dependence of Neutral Amino Acid Uptake

One cornea from each toad was first incubated for the indicated period in a Conway-Ringer solution in which the sodium was replaced by choline (100 mM). The actual sodium concentration in the choline Ringer solution was 10 μM. After the initial incubation period, [^{3}H]-inulin and either L-[^{14}C]-leucine (0.1 mM) L-[^{14}C]-alanine (0.1 mM), or [^{14}C]-AIB (5 μM) was added to the media. The corneas were incubated for an additional 60 minutes and the amount of accumulated amino acid determined. The paired control corneas were handled identically except that the Ringer solution contained sodium (100 mM). The results are expressed as the Mean ± SEM. The number of experiments is shown in parentheses. Paired data was subject to Student's t test: $P < 0.02$ is indicated by ‡, and $P < 0.001$, by §.

Amino Acid	Pre-Incubation Time (hr)	Uptake (pmoles/27 mm^2/hr) Conway-Ringer's	Choline Ringer's	% Change in Amino Acid Uptake
AIB	0.5	0.027 ± 0.003	0.018 ± 0.003	-27.6 ± 14.6 (4)
AIB	2.5	0.034 ± 0.008	0.014 ± 0.004	-55.3 ± 15.2 (6)‡
Alanine	0.5	0.656 ± 0.078	0.340 ± 0.019	-43.8 ± 4.7 (5)§
Alanine	2.5	0.697 ± 0.058	0.115 ± 0.026	-80.0 ± 6.0 (9)§
Leucine	0.5	0.427 ± 0.085	0.365 ± 0.054	2.5 ± 19.6 (4)

anaerobiosis nor cyanide (2 mM) had an apparent effect upon the rate of AIB uptake (Table 6). To rule out the possibility that this apparent lack of effect of cyanide on AIB uptake resulted from paradoxical effects on two systems transporting AIB, we also measured its effects upon the uptake of radioactive alanine and leucine. The uptake of both these amino acids was unaffected by cyanide. Iodoacetate (2 mM) markedly inhibited the uptake of both AIB and alanine but did not reduce leucine uptake by the cornea.

When the cornea was preincubated in sodium arsenite (2 mM) for 30 minutes, the uptake of AIB was increased 45% over the paired controls (Table 7). Even after longer preincubation times (4.5 hrs) the uptake of AIB in the arsenite-treated cornea was sustained at a level significantly greater (16%) than in the paired controls. Uptake studies with radioactively-labeled leucine and alanine showed that arsenite greatly stimulated (56%) the accumulation of leucine but moderately inhibited (36%) the uptake of alanine.

Exposure of the amphibian cornea to arsenite markedly increased the rate of lactate production by the tissue so that there is an increase in the lactate concentration in the bathing media (24). We therefore studied the effects of exogenous sodium lactate upon amino acid uptake by the cornea, using concentrations similar to those normally bathing the endothelial surface of the cornea. As shown in Table 8, D, L-lactate (5 mM) caused a significant increase in the

Table 6: Effect of Metabolic Inhibitors Upon the Accumulation of AIB.

Paired corneas were incubated in modified Conway-Ringer's, pH 8.0; the incubation medium of the experimental cornea of each pair also contained 2 mM of the indicated inhibitor or was rendered anoxic. In the experiments with carbon monoxide, the paired corneas were incubated in Ringer's solution buffered with TES. The experimental corneas were bubbled with 100% CO while the contralateral corneas were bubbled with 100% O_2. After 30 minutes, 5 μC of $[^3H]$-inulin and 1.0 μC of $[^{14}C]$-AIB (0.5 mM), L-$[^{14}C]$-leucine (1 mM), or L-$[^{14}C]$-alanine (1 mM) were added to the media. The corneas were incubated for an additional 60 minutes in this mixture, after which the amount of amino acid accumulated was determined. The amino acid uptake of each cornea exposed to inhibitor was compared to its paired control and the values are expressed as the Mean ± SEM. The number of experiments is shown in parentheses. Paired data were subjected to students t test, $P < 0.01$ is indicated by *, and $P < 0.001$, by §.

Amino Acid	Inhibitor	% Change in Amino Acid Uptake
AIB	Iodoacetate	-48.9 ± 8.5(4) *
Alanine	Iodoacetate	-41.2 ± 3.3(5) §
Leucine	Iodoacetate	8.6 ± 13.5(4)
AIB	Anaerobiosis	4.0 ± 2.5(6)
AIB	Cyanide	-3.1 ± 18.5(5)

(Table 6 continued)

Amino Acid	Inhibitor	% Change in Amino Acid Uptake
Alanine	Cyanide	9.0 ± 13.7(5)
Leucine	Cyanide	11.9 ± 6.5(5)
Leucine	CO	2.9 ± 6.3(14)

Table 7: The Effect of Arsenite Upon Amino Acid Uptake.

Corneas were pre-incubated in modified Conway-Ringer (pH 8.0) containing 2 mM arsenite. The tissues were transferred to media containing 2 mM arsenite, [^{3}H]-inulin, and 1 µC of [^{14}C]-labeled alanine (0.1 mM), leucine (0.1 mM), or AIB (5 µM). The amino acid uptake was determined after one hour incubation in this mixture. Arsenite was omitted from the media bathing paired control corneas. The results are expressed as the Mean ± SEM of the arsenite-treated corneas relative to the paired controls. The number of experiments is shown in parentheses. Paired data was subject to student's t test: $P < 0.05$ is indicated by ϒ, and $P < 0.01$, by *.

Amino Acid	Pre-Incubation Time (hr)	Uptake (pmoles/27 mm^2/hr) Control	Arsenite	% Change in Uptake
AIB	0.5	0.036 ± 0.003	0.046 ± 0.007	44.9 ± 14.2 (5) ϒ
AIB	4.5	0.047 ± 0.005	0.069 ± 0.012	16.1 ± 2.1 (5)*
Leucine	0.5	0.264 ± 0.018	0.404 ± 0.032	56.0 ± 17.1 (5) ϒ
Alanine	0.5	0.714 ± 0.158	0.395 ± 0.038	36.3 ± 15.7 (4)*

Table 8: Effects of Lactate Upon Uptake of Neutral Amino Acids.

The tissues were incubated in modified Conway-Ringer's buffered with bicarbonate (17.7 mM) and containing 1 µC (0.1 mM) of either L-[^{14}C]-alanine, L-[^{14}C]-leucine, or [^{14}C]-AIB and 5 µCi of [^{3}H]-inulin. 5 mM d,l-lactate was added to the experimental corneas while their untreated contralateral cornea served as controls. The pH was maintained at 8.0, and incubations were carried out for one hour. The results are expressed as the Mean ± SEM, relative to the paired control corneas. The number of experiments is shown in parentheses. Paired data were subjected to student's t test: $P < 0.05$ is indicated by Υ, $P < 0.01$, by *.

Amino Acid	Uptake (pmoles/27 mm^2/hr) Control	Lactate	% Change in Amino Acid Uptake
AIB	0.364 ± 0.053	0.414 ± 0.075	10.7 ± 1.1(5)$^{\Upsilon}$
Leucine	0.236 ± 0.054	0.370 ± 0.043	81.2 ± 30.3(5)*
Alanine	0.527 ± 0.069	0.340 ± 0.036	34.6 ± 4.8(4)*

uptake of AIB. When the effects of lactate upon leucine and alanine uptake were separately measured, lactate caused a striking increase in leucine accumulation, and moderately inhibited alanine uptake.

The aqueous humor of almost all vertebrates also contains high concentrations of ascorbic acid which, like lactate, is an effective reducing agent (31). As shown in Table 9, the uptake of both leucine and AIB was significantly stimulated by ascorbate. There was no effect of ascorbate upon alanine uptake. The increase in leucine accumulation was dependent upon the ascorbic acid concentration (Table 10). The approximate Km for ascorbate stimulation of leucine uptake is 0.91 mM. As shown in Table 11, ascorbic acid had no significant effect on the apparent extracellular "space" or the wet weight of the cornea.

We studied the effect of various electron transport inhibitors and uncouplers on ascorbate-stimulated leucine uptake to determine if ascorbate was stimulating leucine uptake by donating electrons to a mitochondrial electron transport chain and thereby stimulating ATP synthesis. As indicated in Table 12, antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), specific inhibitors of the second coupling site between cytochrome b and c (66), had no significant inhibitory effect on ascorbate-stimulated leucine uptake. HOQNO stimulated leucine accumulation in the presence of ascorbate. Cyanide, which inhibits mito-

Table 9: Ascorbate Stimulation of Amino Acid Accumulation by Toad Cornea.

Corneas were incubated in modified Conway-Ringer's containing 1.0 µCi (1 mM) of either L-$[^{14}C]$-alanine, L-$[^{14}C]$-leucine or 1.0 µCi (0.5 mM) $[^{14}C]$-AIB and 5 µCi of ^{3}H -inulin. 20 mM L-ascorbic acid was added to the experimental corneas while their untreated contralateral corneas served as controls. The pH was maintained at 8.0, and incubations were carried out for one hour. The results are expressed as the Mean ± SEM, relative to the paired control corneas. The number of experiments is shown in parentheses. Paired data were subject to students t test. $P < 0.01$ are indicated by *.

Amino Acid	% Change in Uptake
Leucine	34.1 ± 8.7 *
AIB	19.0 ± 5.7 *
Alanine	9.5 ± 18.8

Table 10: Increase in Leucine Accumulation by Toad Cornea in Response to Ascorbic Acid.

Tissues were incubated for one hour in Conway-Ringer's solution, pH 8.0 containing 1 µCi of $[^{14}C]$-leucine (1 mM) and concentrations of ascorbate ranging from 0.2 mM to 20 mM. After one hour the corneas were removed and the radioactivity determined. The control corneas were treated in exactly the same manner except that the incubation media contained no ascorbate. The results are expressed as the Mean ± SEM. The number of experiments is shown parentheses.

Ascorbic Acid Concentration (mM)	% Increase in Leucine Uptake
0.2	6.1 ± 9.0 (6)
2.0	23.8 ± 10.7 (5)
10.0	29.3 ± 3.8 (4)
20.0	34.1 ± 8.7 (6)

Table 11: Effect of Ascorbate on the Wet Weight and Extracellular Space of the Cornea.

Corneas were untreated in modified Conway-Ringer's containing 20 mM L-ascorbic acid, 5 μCi of $[^3H]$-mannitol. At the end of the incubation period, corneas were removed and the radioactivity in both the corneas and the incubation media determined. These values were used to calculate the apparent extracellular space of the corneas. The results are expressed as the Mean ± SEM. The data represent an average of twelve experiments.

	Extracellular Space (μl)		Wet weight (mg)	
Time	Ascorbate	Control	Ascorbate	Control
15 min	2.07 ± 0.15	2.67 ± 0.35	7.97 ± 1.34	8.53 ± 0.58
1 hr	3.41 ± 0.19	3.70 ± 0.19	10.30 ± 0.57	10.57 ± 0.35
4 hr	6.14 ± 0.57	5.55 ± 0.42	14.00 ± 1.21	14.23 ± 1.34

Table 12: Effect of Carbon Monoxide and Electron Transport Inhibitors and Uncouplers on Ascorbate Stimulated Leucine Uptake.

Corneas were first incubated for 0.5 hr. in Conway-Ringer's solution buffered with 17.7 mM $NaHCO_3$, pH 8.0, containing the indicated inhibitor. In the experiments with carbon monoxide, both the control and experimental corneas were incubated with Ringer's solution buffered with TES. 1 µCi (1 mM) of leucine was added and corneas were incubated for an additional hour. Control corneas were treated identically except that the medium was bubbled with 99% O_2, 1% CO_2 and no inhibitors were added. The results are expressed as the Mean ± SEM. The number of experiments are shown in parentheses. Paired data were subject to students t test. $P < 0.05$ is indicated by $^{\Psi}$, and $P < 0.01$ by *.

Inhibitor	% Change in Amino Acid Uptake
CO	-25.6 ± 4.2(6) *
DNP	-6.5 ± 19.0(6)
HOQNO	29.0 ± 8.8(6) $^{\Psi}$
Antimycin A	17.9 ± 16.9(6)
Oligomycin	7.5 ± 10.6 (6)
Cyanide	3.1 ± 5.2(7)

chondrial cytochrome oxidase, was also without effect. Dinitrophenol, an uncoupler of oxidative phosphorylation, and oligomycin, which inhibits the mitochondrial ATPase, were also ineffective when tested against the ascorbate effect upon amino acid uptake.

Carbon monoxide is the only agent we found effective as an inhibitor of the ascorbate stimulation of leucine uptake. Paired corneas were incubated in media containing ascorbic acid (2 mM) and ^{14}C-leucine (1.0 mM). One cornea from each pair was bubbled with 100% carbon monoxide while the contralateral cornea was incubated with 100% oxygen. We found that leucine uptake was inhibited by 26 ± 4% in those corneas exposed to carbon monoxide (Table 12). In our earlier experiments, in which one cornea of each pair was treated with ascorbic acid, the increment in leucine uptake was 34 ± 9% (Table 9). These data indicate that leucine is accumulated by two systems, one of which is stimulated by ascorbate, and is inhibited by carbon monoxide. This conclusion is further supported by the fact that leucine accumulation in the absence of ascorbate is unaffected by carbon monoxide (Table 6).

We studied the effects of exogenous ATP upon amino acid accumulation to further explore the possibility that the stimulation of amino acid uptake by ascorbate and lactate was due to an increase in cellular ATP levels. The data in Table 13 shows that ATP significantly stimulates

Table 13: Effect of ATP on Amino Acid Accumulation by Toad Cornea.

Tissues were incubated with Conway-Ringer's, pH 8.0, containing the indicated concentration of ATP and the appropriate ^{14}C-labeled amino acid. The incubated medium for the contralateral control corneas contained no ATP. The number of experiments is indicated in parentheses.

Amino Acid	ATP Concentration	% Change in Amino Acid Uptake
Alanine	0.1 mM	26.8 ± 17.9 (6)
	1.0 mM	86.0 ± 24.4 (6)
Leucine	0.1 mM	7.4 ± 13.7 (6)
	1.0 mM	100.2 ± 30.2 (5)
AIB	1.0 mM	-1.9 ± 16.9 (6)

the uptake of both alanine and leucine by the toad cornea but has no significant effect upon AIB accumulation. These results indicate that ATP is probably stimulating cellular metabolism and, therefore, the metabolism of these amino acids. Increased metabolism of leucine and alanine would lower their intracellular levels, accelerating the accumulation of these amino acids. ATP, unlike ascorbate and lactate which specifically stimulates only leucine accumulation, has similar stimulatory effects on leucine and alanine accumulation. Therefore, we conclude that ATP plays no direct role in the action of lactate and ascorbate upon amino acid transport.

It has been reported that N^6,2'-O-dibutyryl, 3',5'-cyclic adenosine monophosphate (dibutyryl cAMP) stimulates ion transport across both the amphibian (67) and rabbit cornea (68). We therefore examined the effects of adenosine 3',5'-monophosphate (cAMP) and its dibutyryl derivative on amino acid accumulation. We found that incubation of the toad cornea for a period of one hour with either of these cyclic nucleotides had no significant effect on the accumulation of AIB, leucine or alanine. Amino acid uptake was significantly increased (Table 14) when corneas were preincubated for two hours with dibutyryl cAMP. Dibutyryl cAMP significantly increased the uptake of alanine, leucine and AIB by the toad cornea. The increase in accumulation was greatest for AIB and leucine, the uptake of these

Table 14: Stimulation of Amino Acid Accumulation by Toad Cornea in the Presence of $N^6,O^{2'}$ Dibutyryl 3',5'-Cyclic Adenosine Monophosphate.

Corneas were first incubated for 2.0 hrs. in modified Conway-Ringer's, pH 8.0, containing the indicated additions. After 2 hrs. of incubation 1 µCi of the appropriate ^{14}C-labeled amino acid (1.0 mM leucine, alanine, or 0.5 mM AIB) and 5.0 µCi ^{3}H-mannitol were added to the incubation media and the tissues were incubated for an additional hour. The number of experiments is shown in parentheses. Results are expressed as the Mean ± SEM.

Amino Acid	Uptake (pmoles/27 mm^2/hr)		
	Control	Dibutyryl cAMP	Dibutyryl cAMP Ouabain
Alanine	2.79 ± 0.32(6)	4.46 ± 0.40(6)	2.38 ± 0.32(6)
Leucine	1.23 ± 0.28(6)	3.31 ± 0.63(6)	1.05 ± 0.12(6)
AIB	1.14 ± 0.41(6)	3.53 ± 0.48(6)	2.45 ± 0.54(6)

amino acids being stimulated 156 ± 49% and 186 ± 33%, respectively. Alanine uptake was also significantly increased by 61 ± 11%. As shown in Table 14, ouabain completely blocks dibutyryl cAMP stimulated amino acid accumulation. Ouabain is ineffective in blocking amino acid accumulation in the absence of dibutyryl cAMP. One millimolar cAMP was ineffective in stimulating leucine, AIB, and alanine accumulation.

A number of clinically important ophthalmic drugs are administered topically, with the result that the cornea (at least, the epithelial surface) is repeatedly exposed to high concentrations of these agents. We tested the effects of several of the more commonly used drugs upon amino acid uptake. Echothiophate iodide (Phospholine iodide), an irreversible inhibitor of cholinesterase, is used in the treatment of glaucoma (69). Patients develop cataracts in many cases after 6 months or more of treatment with this drug (70). In the presence of Phospholine iodide (2.5 mM) AIB uptake was reduced 55.6 ± 9.2% as compared to control corneas. This concentration of Phospholine iodide is equivalent to two drops of 0.25% Phospholine iodide placed on the epithelial surface of the cornea (in the tear film), a commonly used therapeutic procedure.

Eserine (physostigmine) is a reversible inhibitor of cholinesterase and is also used in the treatment of glaucoma. AIB accumulation by the toad cornea was not significantly

reduced by eserine in concentrations ranging from 0.01 µM to 2 mM. Atropine, a plant alkaloid, is an effective competitive antagonist of acetylcholine at the membrane receptors on smooth muscle and other cells. It use in ophthalmology is to dilate the pupil. 2 mM atropine sulfate did not significantly reduce AIB uptake by the cornea. 2 mM methacholine (Mecholyl), an analog of acetylcholine, also had no significant effect upon AIB uptake.

We also examined the effects of these drugs on the uptake of 3-0-methyl-D-glucose, a non-metabolizable analog of glucose. The uptake of 3-0-methyl-D-glucose was 0.010 ± 0.001 pmoles/27 mm^2/2.5 hr. in Conway-Ringer's solution. When the 2.5 hr. incubation was carried out in the presence of Phospholine iodide, 3-0-methyl-D-glucose uptake was 0.010 ± 0.001 pmoles/27 mm^2/hr. This indicates that Phospholine iodide has a rather specific effect upon amino acid accumulation.

CHAPTER IV

ION TRANSPORT

The ion transport properties of the amphibian cornea have been studied almost exclusively in the frog, *Rana catesbeiana*. This tissue transports chloride from the endothelial to the epithelial surface (22) and, to a small extent, sodium in the opposite direction (23). The ion transport properties of the cornea of the toad, *Bufo marinus*, have not been investigated. Table 17 indicates the values for the transport of sodium and chloride by the short-circuited toad cornea. The net flux of chloride is from the endothelium to the epithelium while the net movement of sodium occurs from the epithelium to the endothelium. It can be seen that in the toad cornea, unlike the frog, the transport of sodium is almost as great as the transport of chloride.

Our finding that arsenite markedly increases amino acid accumulation by the toad cornea recalls the observation by Zadunaisky that arsenite causes a large transient stimulation of chloride transport in the frog cornea (24, 27). We, therefore, examined the possibility that arsenite, lactate, and ascorbate might also stimulate ion transport in the toad cornea.

We mounted freshly excised toad corneas in typical Ussing-style Lucite chambers as described in Materials and Methods. The short-circuit current (SCC) was measured continuously except for brief periods in which the spontaneous potential difference (PD) was measured. The toad cornea developed a PD of from 5-12 mV (epithelial or tear surface negative) and the SCC amounted to 2-5 μAmp/cm^2. The addition of 1 mM sodium arsenite to the bathing solutions caused a small, but reproducible, increase in SCC and PD. D,L-Lactate (5 mM) had a similar effect upon the electrical parameters (Figure 8). The addition of 2 mM ascorbate to the chamber bath was followed by a remarkable rise in both SCC and PD. This increment, which was achieved in two to three minutes, was sustained for up to one hour and was reversed immediately by washing out the ascorbic acid (Figure 9). A similar effect is obtained in the cornea of the frog, Rana catesbeiana.

We next sought to determine whether the effects of ascorbic acid were related to its redox state. Ascorbic acid was oxidized immediately before use by the method of Frey, Pitts and Askari (65). The addition of dehydroascorbic acid (DHA) to the bathing media had a negligible effect upon the electrical parameters. In five experiments the SCC and PD were not significantly altered 12 minutes after the addition of 2 mM DHA (table 15 and Figure 10). The very small effects observed in some

Figure 8: Corneas were mounted in Ussing-style chambers and bathed on both surfaces with Ringer's solution. At the indicated times 1 mM Na arsenite, 5 mM D,L-lactate or 2 mM L-ascorbic acid were added. The SCC was monitored continuously except for brief periods in which the spontaneous potential difference was determined.

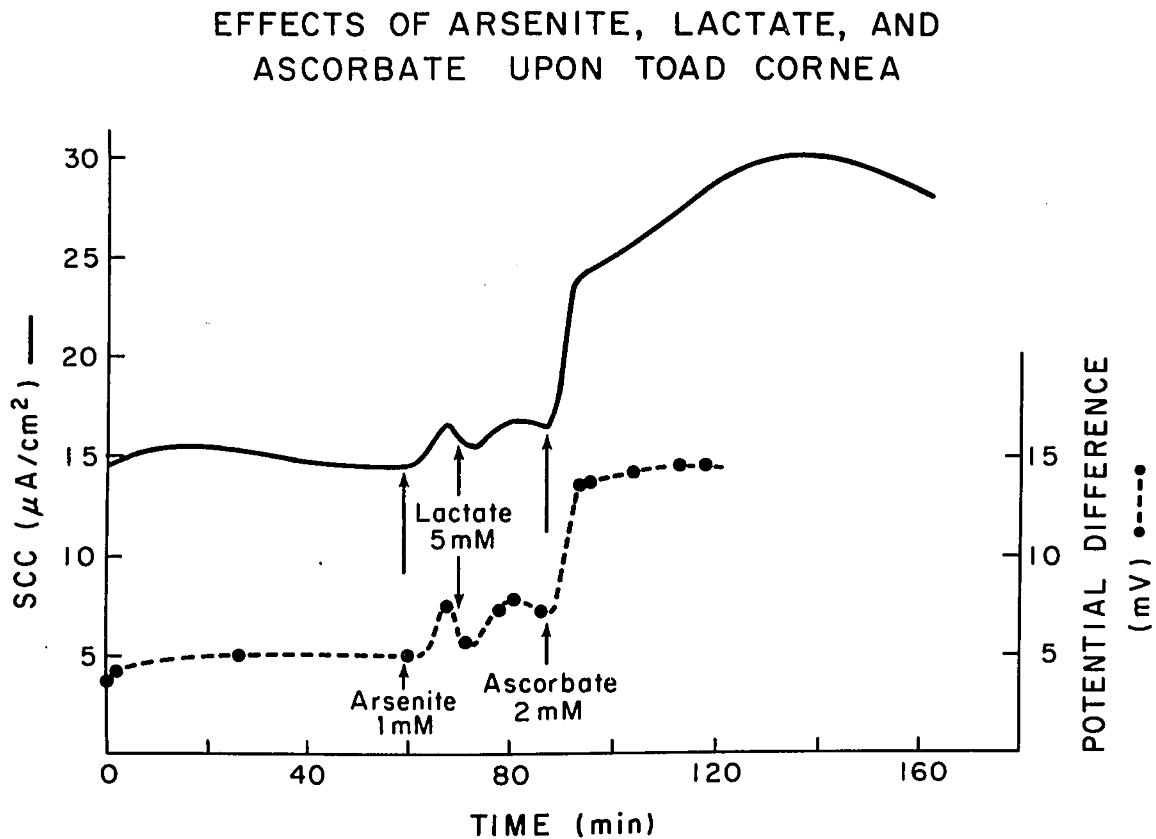


Figure 9: Corneas were mounted in Lucite chambers and were allowed to stabilize for 30-45 minutes. 2 mM ascorbate was then added to both bathing solutions. After 15 minutes the ascorbate was washed out and fresh Ringer added to the chamber reservoirs.

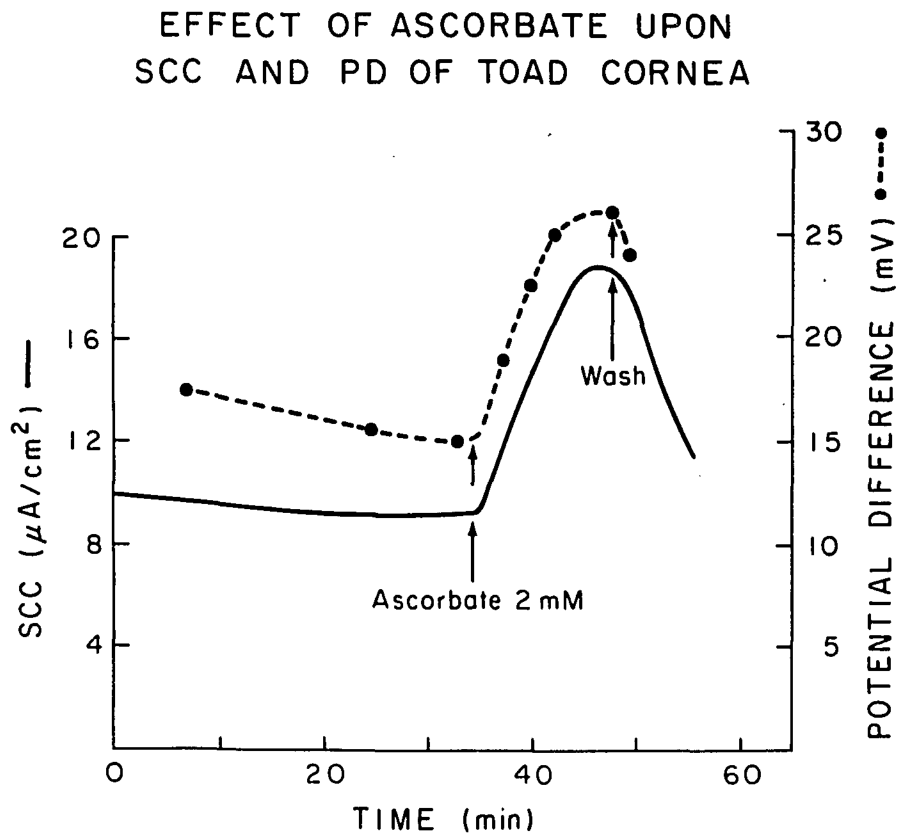


Figure 10: Corneas from the toad, Bufo marinus, were mounted in Ussing-style Lucite chambers and allowed to stabilize. 2 mM ascorbate acid was added to both bathing solutions of one cornea while 2 mM dehydroascorbic acid was added to the solutions bathing the contralateral cornea.

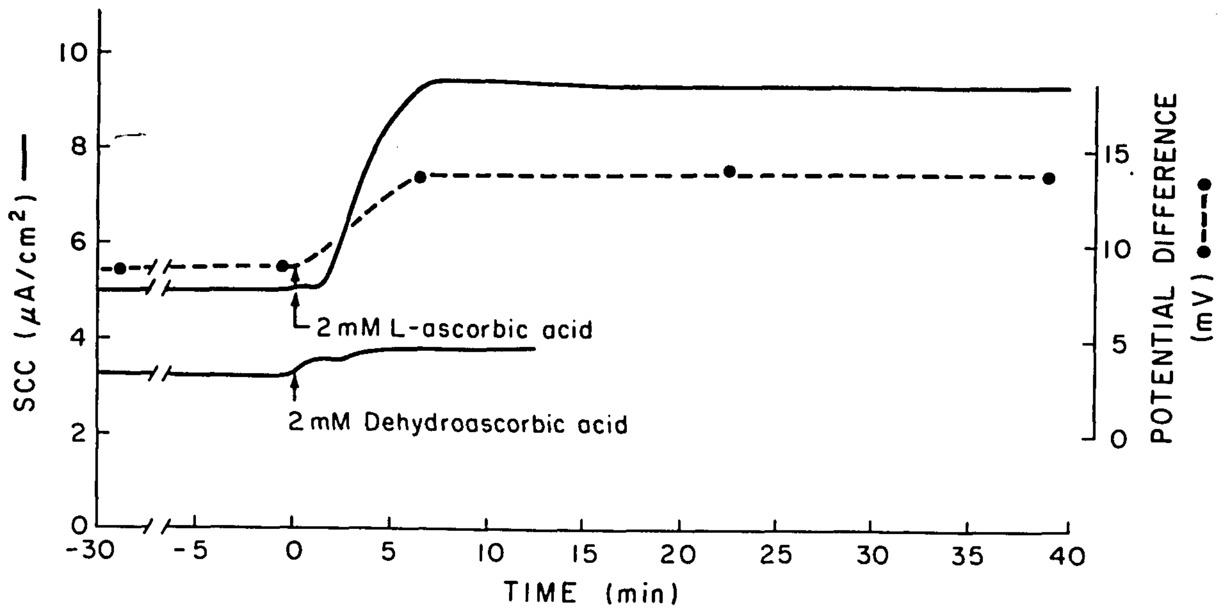


Table 15: Effects of Ascorbate Analogs Upon SCC and PD of Toad Cornea.

Corneas were mounted in Lucite chambers and bathed in Ringer's solution. The increment in SCC and PD were measured 12 minutes following the addition of 2 mM ascorbic acid, dehydroascorbic acid, or D-isoascorbic acid to each bath. The values for each compound are the Mean ± SEM of five different corneas.

	Increment	
Addition	SCC (uA/cm^2) (% increase)	PD (mV) (% increase)
Ascorbic Acid	5.9 ± 0.8 (205%)	10.2 ± 1.9 (136%)
Dehydroascorbic Acid	0.4 ± 0.5 (8%)	0.8 ± 0.7 (9%)
D-isoascorbic Acid	6.1 ± 0.7 (192%)	8.8 ± 0.5 (120%)

instances may have represented the effects of traces of reduced ascorbate undetectable by our method of analysis (thin-layer chromatography). D-isoascorbic acid, the epimer of ascorbic acid, was as effective as ascorbic acid in stimulating the SCC and PD.

Table 15 also indicates that both ascorbic acid and D-isoascorbate decreases the resistance of the cornea. Corneal resistance is determined by the ratio PD/SCC or E/I. In eight experiments, corneal resistance was decreased by 24.8 $\pm$ 4.6%(8) in the presence of ascorbic acid.

Although the concentration of ascorbic acid in the aqueous humor of most mammalian species is about 2 mM, the concentration in the amphibian aqueous humor is only 0.1 to 0.2 mM (31). We next sought to determine if ascorbic acid in concentrations normally found in amphibian aqueous was effective in promoting ion transport. We found that the addition of ascorbic acid in concentrations as low as 10 uM to both surfaces of the cornea caused a significant increase in both the PD and SCC. We next tested the dose-response relationship of ascorbate by the sequential addition of increasing amounts of ascorbic acid to the cornea. The response of each cornea at each concentration was related to the maximal response of the same cornea to ascorbate, which was taken as the SCC in the presence of 2 mM ascorbic acid. In five corneas, the addition of 0.1 mM ascorbic acid was followed by an increase

in SCC amounting to 67% of the maximal response subsequently obtained in the same cornea (Table 16). The increase in SCC and PD was dependent upon the concentration of ascorbic acid, and all concentrations of this metabolite reduced the resistance of the cornea.

Using ^{24}Na and ^{36}Cl, we next sought to determine if chloride was increasing the SCC by stimulating sodium or chloride fluxes. As shown in Table 17, the primary effect of ascorbate is to greatly enhance the flux of chloride from the endothelial to epithelial surfaces. The change in the net flux of chloride amounted to a 285% increase.

We also studied the effects of various inhibitors and uncouplers of mitochondrial electron transport to determine if ATP synthesis was directly mediating the ascorbate effect upon ion transport. Rotenone, which blocks the mitochondrial electron transport system at the level of the reduction of cytochrome b, did not inhibit the response of the cornea to the subsequent addition of ascorbate (2 mM).

Cyanide (2 mM) blocks the mitochondrial electron transport system at the level of the reduction of cytochrome (a.a3). When cyanide is added to the epithelial bathing solution it causes both the SCC and PD to drop to zero. The addition of cyanide at the peak of the SCC response to 2 mM ascorbate caused a 42.1 ± 7.9% (3) decrease in the PD and a 42.9 ± 8.7%(3) decrease in the SCC. These electrical parameters do not, however, drop to zero

Table 16: Effects of Increasing Concentrations of Ascorbate on the SCC and PD of Toad Cornea.

Corneas were mounted in Lucite chambers as described in Materials and Methods, and allowed to stabilize for 30-45 minutes. Doses of ascorbate ranging from 10 µM to 5 mM were added to both bathing solutions. The short-circuit current was measured continuously except for brief intervals in which the spontaneous potential difference was measured. The response to 2 mM ascorbate was maximal and is taken as 100%.

Concentration of Ascorbate (mM)	% of Maximal Response	
	SCC (uA/cm^2)	PD (mV)
0.10	67%	71%
0.50	81%	83%
1.0	89%	84%
2.0	100%	100%

Table 17: Effects of Ascorbic Acid Upon Fluxes of Sodium and Chloride in Toad Cornea.

Corneas, mounted in Lucite chambers and bathed in a modified Conway-Ringer solution (pH 7.4), were allowed to stabilize for 30-45 minutes. Either ^{36}Cl (4.3 mCi/mg) or ^{22}Na (5 mCi/mg) was added to the solution bathing one surface of the cornea and 2.0 ml samples taken at three 20 minute intervals from the bath on the opposite side of the tissue. Ascorbate (2 mM) was then added to both bathing solutions and samples removed for radioactive measurement for four additional intervals. The samples were counted in a triton-toluene scintillation fluid in a Packard 3320 liquid scintillation spectrometer. The results are given as the Mean ± SEM and the number of corneas studied in parentheses. Unpaired data was subjected to student's t test. $P < 0.025$ is indicated by **, $P < 0.05$ is indicated by γ.

Unidirectional Fluxes (in Eq x cm^{-2}xhr^{-1})

Isotope	Flux	Control	Ascorbate	Ascorbate-Induced Increment
^{36}Cl	Endo→Epi	0.272 ± 0.020(4)	0.542 ± 0.089(4)	0.270 ± 0.091**
	Epi→Endo	0.197 ± 0.023(4)	0.251 ± 0.036(4)	0.054 ± 0.043
	Net	0.076 ± 0.031(4)	0.291 ± 0.082(4)	0.215 ± 0.088 γ
^{22}Na	Endo→Epi	0.105 ± 0.007(4)	0.133 ± 0.030(4)	0.028 ± 0.031
	Epi→Endo	0.159 ± 0.018(4)	0.214 ± 0.040(4)	0.055 ± 0.044
	Net	0.054 ± 0.020(4)	0.081 ± 0.040(4)	0.027 ± 0.045

following cyanide (Figure 12), but remain near the levels prior to the addition of ascorbate. Cyanide is only effective in inhibiting ion transport when it is added to the epithelial bathing solution.

Dinitrophenol (DNP) is an uncoupler of mitochondrial oxidative phosphorylation. One mM DNP added to the endothelial bathing solution causes both the PD and SCC to drop to zero (Figure 13). However, the SCC and PD respond to the subsequent addition of 2 mM ascorbate by increases similar to those seen in the untreated cornea. It appears that DNP does enter the cornea and does eliminate ion transport in the absence of ascorbate. This indicates that ion transport across the toad cornea is dependent upon ATP production in the absence of ascorbate. DNP does not block ascorbate-stimulated ion transport and therefore we conclude that, like the transport of leucine, there are two modes of chloride transport. One of these is blocked by metabolic inhibitors and the other, insensitive to these inhibitors, is responsive to the presence of ascorbic acid (the latter is sensitive only to carbon monoxide).

We also found that the addition of 1 mM ATP to the solutions bathing the cornea had no effect on either the PD or SCC. The fact that ATP stimulates the uptake of metabolizable amino acids (Table 13) indicates that the nucleotide does penetrate the cornea. These results provide further evidence that ATP synthesis is not the

Figure 11: 30 µM rotenone was added to both solutions bathing the cornea. 30 minutes following the addition of rotenone, 2 mM ascorbate was added to both bathing solutions. The SCC was monitored continuously except for brief periods during which the spontaneous potential difference was determined.

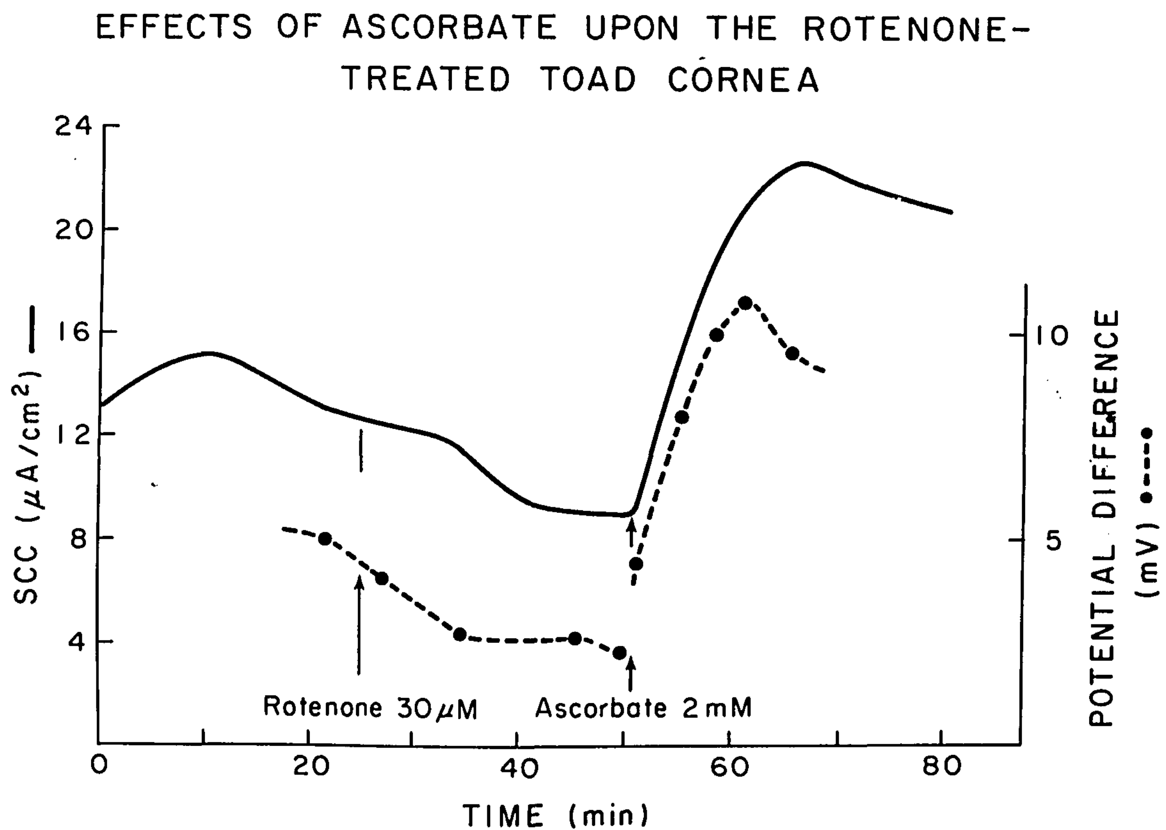


Figure 12: Corneas were mounted in Lucite chambers, bathed on both surfaces with a modified Conway-Ringer and allowed to stabilize. 2 mM ascorbic acid was added to the epithelial bathing solution. At the peak of the response to ascorbate 2 mM Na cyanide was added to the epithelial bathing solution.

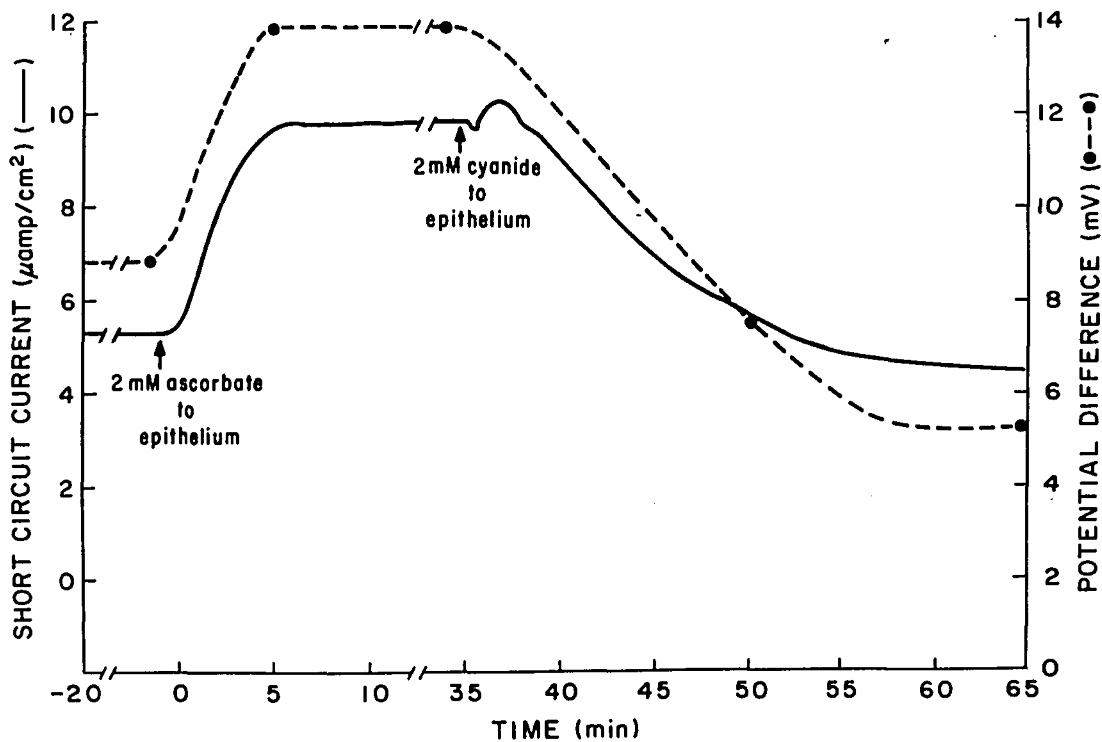
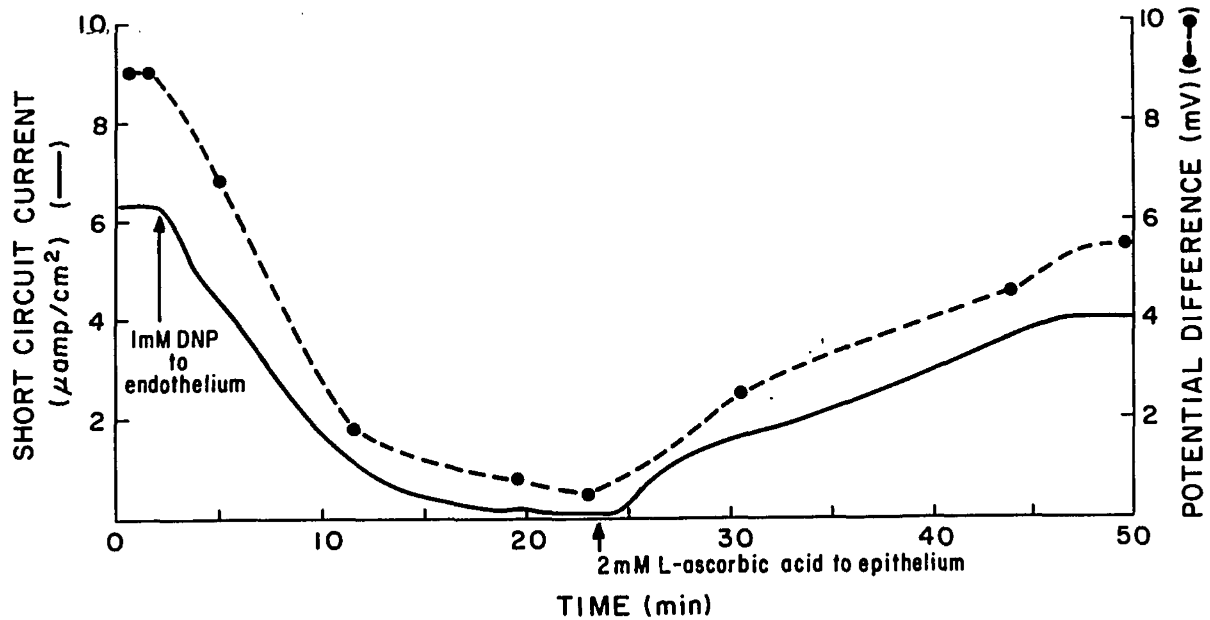


Figure 13: Corneas, mounted in Ussing-style chambers, were allowed to stabilize. 1 mM DNP was added to the endothelial bathing solution. The SCC and PD both dropped to zero at which time 2 mM ascorbic acid was added to the epithelial bathing solution.



rate-limiting step in the stimulation of ion transport by ascorbate.

CHAPTER V

D I S C U S S I O N

A. Extracellular Space of the Cornea

The measurement of the extracellular space of the *in vitro* toad cornea using inulin or mannitol indicates that mannitol equilibrates in the extracellular water much more rapidly than inulin and is therefore the better indicator of the extracellular space of the cornea in short-term experiments. After four hours of incubation in Conway-Ringer's solution, the inulin and mannitol spaces are equal, indicating that mannitol does not enter the intracellular space. It is generally considered that inulin is the better extracellular space indicator because mannitol is not completely excluded from the intracellular space. However, studies in the canine small intestine showed that, although mannitol equilibrates more rapidly in the extracellular space than inulin, it never enters the cell water (71). Page, in a detailed study of the extracellular space of cat papillary muscle, found that the mannitol space is considerably greater than the inulin space despite the fact that both appear to be excluded from the cell water (72). Both studies concluded that a part of the extracellular space was only slowly accessible to inulin. Study of the extracellular space of the canine carotid artery wall, using

both electronmicrographic and tracer techniques, showed that inulin did not enter all the extracellular water (73). Our data indicate that inulin gains access to all of the extracellular water in the cornea but that this occurs at a slower rate for inulin than for mannitol. It has been shown that the corneal epithelium behaves primarily as a relatively impermeable barrier, and that most substances enter the cornea across the endothelial surface. Kim et al. have shown that the permeability of the endothelium to non-electrolytes is inversely proportional to their molecular weight (7). Therefore, it would be expected that inulin, with a molecular weight of approximately 5000, would diffuse across the corneal endothelium much more slowly than mannitol (molecular weight = 181). This may account for the nonlinear nature of α-aminoisobutyric acid (AIB) uptake vs. time when the correction for extracellular AIB is determined by the inulin space (Figure 4). The more rapid initial uptake rate of AIB during the first hour (Figure 4) is inconsistent with the rate of AIB uptake during the following three hours and also with the uptake curve for AIB when mannitol is used to measure the extracellular space (Figure 3). This initial rapid uptake of AIB can be accounted for by the fact that AIB is moving across the endothelium more rapidly than inulin and is therefore equilibrating with the extracellular water more rapidly. The concentration of AIB in the extracellular space is, therefore, significantly creater than that of

inulin, and the correction for the extracellular AIB is spuriously low. Mannitol, with a molecular weight more closely approximating that of AIB, presumably enters the extracellular water at approximately the same rate as AIB. Another indication that mannitol equilibrates in the extracellular water very rapidly is the observation that the increase in mannitol space with time parallels the increase in the wet weight of the cornea.

Removal of sodium from the incubation media intially causes a slight decrease in the extracellular space of the toad cornea. After 2.5 hours of incubation in sodium-free Ringer's solution, the extracellular space of the cornea increases significantly. Candia has found that after two hours of incubation of the bullfrog cornea in sodium-free Ringer's solution there is a large increase in both unidirectional chloride fluxes to approximately 80 uA/cm^2 (25). These data suggest that removal of sodium increases corneal permeability and promotes corneal swelling.

B. Amino Acid Accumulation Studies

AIB enters the *in vitro* toad cornea across the endothelial surface. This conclusion is in agreement with the studies of Thoft and Friend who demonstrated that AIB enters the *in vivo* rabbit cornea across the endothelium, the epithelium being relatively impermeable to this amino acid (38). This result is also in agreement with the finding

that glucose enters the cornea through the endothelium (5, 6).

The *in vitro* toad cornea exhibits saturable carrier-mediated accumulation of AIB. This amino acid is generally considered to be a substrate for the transport system responsible for the sodium-dependent uptake of glycine and alanine (the A system) (44), and the accumulation of radioactive AIB by tissues is often interpreted as a measure of this single transport process (41). However, in the toad cornea AIB uptake is apparently mediated by at least two separate systems. The uptake of this amino acid is reduced only about 60% by the addition of high concentrations of alanine. Glycine, also a preferred substrate for the A-transport system (43), inhibits AIB uptake about 40%. Leucine, as well as isoleucine and valine, are transported by the L system, which does not accumulate AIB in most tissues (43). The presence of leucine in the medium, which has no effect upon alanine uptake in the toad cornea, reduces the uptake of AIB by about 30%. This result indicates that a large portion of the AIB is accumulated by a mechanism normally transporting leucine, presumably the L system. The effects of alanine, glycine, and leucine upon AIB uptake are related to their concentrations, with a maximum inhibition of AIB uptake occurring at a five-fold concentration of each of the natural amino acids. The inhibition of AIB uptake by alanine exhibits a slight

decrease at a concentration of 5 mM alanine. Because the inhibition achieved with 2.5 mM and 10 mM alanine is identical we feel that maximal inhibition of AIB occurs at a concentration of 2.5 mM alanine.

The inhibitory effects of unlabeled leucine upon AIB uptake are additive with those of glycine or alanine. The presence of both leucine and glycine does not, however, completely block AIB accumulation by the cornea. AIB uptake in the presence of 20-fold concentrations of both leucine and glycine amounts to 26% of the control value. This indicates the participation of yet another transport system in the accumulation of AIB by the toad cornea. We found that the further addition of serine (10 mM) to the incubation medium containing saturating concentrations (1) mM) of leucine and glycine reduces this small residual AIB uptake by approximately 35%. These data suggest the participation of a third transport system, which is competitively inhibited by serine, in the accumulation of AIB. This third system may be the ASC-system originally described by Christensen, Liang and Archer in the Ehrlich cell (46). The ASC-system transports alanine, cysteine and serine but glycine is excluded from transport by this system. In our studies we found that the degree of inhibition of AIB uptake in the presence of 10 mM alanine plus 10 mM leucine is not significantly different from the inhibition of AIB uptake in the presence of 10 mM glycine

plus 10 mM leucine plus 10 mM serine (P = .20). On the other hand, the inhibition of AIB uptake by 10 mM alanine plus 10 mM leucine was significantly greater ($P<0.005$) than the inhibition of AIB uptake by 10 mM glycine plus 10 mM leucine. We also found that alanine inhibited AIB uptake to a greater extent than glycine. Transport via the ASC-system has been found to be sodium-dependent in all tissues studied (46). When corneas were incubated in the presence of high concentrations of alanine and leucine in media with or without sodium we found no differential effects upon AIB uptake. This indicates that this third system involved in AIB accumulation is not sodium-dependent. In summary, our results indicate that accumulation of AIB by the toad cornea is mediated by three systems. The third system transports alanine and AIB but glycine and leucine are excluded from transport by this system. In this sense, it closely resembles the ASC system. This third system differs from the ASC system in that it is not sodium-dependent.

These results show that AIB uptake does not represent the activity of one homogeneous transport system in the cornea and therefore uptake data obtained with this amino acid must be interpreted with this serious limitation in mind.

The accumulation of amino acids by the A system in most tissues is coupled to the movement of sodium into

the tissue and is inhibited by lack of sodium in the medium (41, 42). A significant portion of the AIB uptake by the toad cornea is maintained when sodium is replaced with choline. The degree of inhibition is related to the length of time that the cornea is incubated in choline Ringer's, but never exceeds approximately 55%. This degree of inhibition is approximately the same as that obtained using alanine as a competitive inhibitor of AIB uptake. We measured the effects of sodium replacement upon the uptake of radioactive alanine and found that the degree of inhibition of uptake of this amino acid is also related to the length of the preincubation period in sodium-free Ringer. Because the relative inhibition of alanine uptake is approximately 50-75% greater than that of AIB, and because replacement of sodium has no effect upon the accumulation of radioactive leucine, we interpreted these data as further evidence that AIB is transported by systems other than the sodium-dependent A system. The time-dependent nature of the inhibition, and the fact that alanine uptake is not completely inhibited, may be due to the fact that these processes, coupled to sodium, are located in a portion of the cornea which retains significant amounts of sodium. For example, these transport processes may be located at the basal margin of the epithelium, in which case the time-dependent elution of extracellular sodium from the underlying stroma would require a long period to

reduce the local concentration of sodium to inhibitory levels.

When the effects of various metabolic inhibitors upon AIB uptake were studied, it was found that only iodoacetate was effective in inhibiting the uptake of this amino acid. Iodoacetate inhibits the enzyme 3-phosphoglyceraldehyde dehydrogenase thus blocking glycolysis. Because iodoacetate only inhibits the accumulation of AIB and alanine, we conclude that glycolysis provides part of the energy for accumulation of amino acids via the sodium-dependent transport systems. Schafer and Heinz have found that the potential energy of the sodium-gradient is not sufficient to account for all the energy required for AIB accumulation by the Ehrlich cell (52). In the toad cornea the energy derived from glycolysis appears to be necessary for maximal accumulation of amino acids via systems generally considered to be sodium-dependent. Glycolysis evidently is not providing the energy required to maintain the sodium-gradient across cells because our studies with ouabain and the removal of sodium indicate that the sodium-gradient would not be reduced significantly during a one hour incubation.

An unexpected and remarkable finding was the substantial stimulation of AIB uptake by arsenite. Amino acid uptake, initially increased by approximately 50%, was maintained at a significantly higher rate compared to con-

trols even after a preincubation period of 4.5 hours in arsenite. This sustained increase in amino acid uptake following arsenite is similar to the effects of this agent upon chloride transport across the bullfrog cornea (24). We further found that arsenite markedly stimulates the uptake of radioactive leucine (56%), while the uptake of radioactive alanine is reduced (36%) by this agent. Apparently the large increment in AIB uptake is an expression of the stimulation of the system normally transporting leucine and AIB and a lesser inhibition of the system(s) normally transporting alanine and AIB.

Arsenite causes an accumulation of lactate in the media bathing the cornea (27), presumably by inhibiting tissue pyruvate dehydrogenase (76). Pyruvate dehydrogenase is a multi-enzyme system responsible for the conversion of pyruvate to acetyl CoA. One component of this complex is dihydrolipoate dehydrogenase. Arsenite reacts with the sulfhydryl residues on dihydrolipoate and thereby prevents the reoxidation of lipoate. This inhibition blocks the further metabolism of pyruvate furnished by the glycolytic pathway and causes the accumulation of pyruvate. The presence of lactic dehydrogenase would permit the concomitant increase in lactic acid. The addition of D,L-lactate to the bathing media of the untreated cornea gives results very similar to those found in the arsenite treated cornea: leucine uptake is almost doubled, while alanine accumula-

tion is significantly reduced. These data indicate that the effects of arsenite upon amino acid uptake by the cornea may be mediated by a rise in tissue lactate levels. They also suggest that the high concentration of lactic acid normally present in the aqueous humor (31) may have significant effects upon the transport properties of the _in vivo_ cornea.

Ascorbic acid is another reducing agent found in rather high concentrations in the aqueous humor. As indicated in Table 9, ascorbate causes a concentration-dependent increase in leucine accumulation by the _in vitro_ toad cornea. This increase is unaffected by classical inhibitors of mitochondrial electron transport, indicating that this increase in transport is not due to an increase in ATP synthesis. This conclusion is further substantiated by the fact that exogenous ATP stimulates the transport of metabolizable amino acids and has no differential effect on the various systems responsible for amino acid accumulation. There are only two other reports of stimulation of transport by exogenous ATP known to the author. Kidder (75) found that exogenous ATP causes a transient increase in acid secretion by the frog gastric mucosa. This effect of ATP was correlated with a reduction of cytochrome c. Gerencser and Armstrong (76) showed that ATP, when added to the mucosal bathing solution, stimulates the mucosal to serosal sodium flux across bullfrog small intestine.

The simplest explanation for the effect of ATP in these tissues is that ATP is entering the cells. Although these authors have not shown this to be the case, there are other reports of penetration of ATP into cells (77, 78).

Kaback and his colleagues have described an electron transport-dependent stimulation of amino acid and sugar uptake in membrane vesicles prepared from bacteria (79-83). In vesicles prepared from E. coli they found that D-lactate, D,L-2-hydroxybutyrate, succinate, ascorbate, and L-lactate stimulate the uptake of amino acids and sugars through a membrane-bound D-lactic dehydrogenase coupled to an electron transport chain (83). ATP and phospho-enol-pyruvate were effective in stimulating amino acid and sugar uptake and the system was not inhibited by high concentrations of arsenate or oligomycin, indicating that the electron transport chain did not involve oxidative phosphorylation (80). The mechanism proposed by these workers (79) depicts the carriers for amino acids and sugars as electron transfer immediates which undergo reversible oxidation and reduction. In the oxidized state, the "carrier" has a high affinity site for the ligand (amino acid or sugar) which it binds at the exterior surface of the membrane. Electrons coming from one of the electron donors (lactate, ascorbate, etc.) reduce a critical disulfide bond in the carrier resulting in a conformational change. This reduces the affinity of the carrier for its ligand and the

ligand is released on the interior surface of the membrane. Kaback further proposes the presence of more than one electron transfer carrier because amino acid uptake competition experiments indicate the presence of specific transport systems.

A similar system may be responsible for the ascorbate-stimulated accumulation of amino acids by a system whose specificity is similar to that of the L-system. The lack of effect of ATP, oligomycin, and dinitrophenol indicates that oxidative phosphorylation is not involved in the stimulation of amino acid transport caused by ascorbate. The capacity of carbon monoxide to inhibit ascorbate stimulated leucine uptake indicates that microsomal electron transport may be involved. Cytochrome P450, a microsomal cytochrome, is inhibited by carbon monoxide but is unaffected by cyanide and other inhibitors of mitochondrial electron transport (84). 2-Heptyl-4-hydroxyquinoline-N-oxide (HOQNO) probably stimulates leucine accumulation by blocking the reverse flow of electrons from ATP through the mitochondrial electron transport chain. This inhibition of reverse electron flow leads to higher levels of ATP, which stimulates leucine accumulation. We therefore suggest that lactate and ascorbate, compounds found in high concentrations in the aqueous humor of most vertebrates, play a specific role in corneal amino acid transport. These compounds are both reducing agents and our

evidence indicates that they act as electron donors to an extramitochondrial electron transport chain. Reduction of the carrier protein of an amino acid transport system which closely resembles the L system stimulates accumulation via this system. Ascorbate is actively transported across the ciliary processes at the expense of considerable metabolic energy and it may be that this is the means by which the vascular ciliary body transfers energy to the avascular cornea. This energy, in the form of reduced ascorbate, helps to provide the cornea with amino acids for protein synthesis.

We have found that dibutyryl cAMP stimulates the uptake of AIB, alanine, and leucine. This effect is not specific for one amino acid transport system because we have shown that alanine is transported by the sodium-dependent A system while leucine is transported by the sodium-independent L system. Because ouabain inhibits the dibutyryl cAMP-stimulated component of amino acid accumulation we suggest that dibutyryl cAMP stimulates amino acid transport by modifying a sodium-sensitive intermediate involved in amino acid transport. We have also found that the stimulation of amino acid accumulation by dibutyryl cAMP is not immediate. Preincubation of the tissue for two hours in the presence of this agent is required before any effect on amino acid accumulation is seen. These results are consistent with those of Weiss et al. (85).

These researchers found that 1 mM dibutyryl cAMP stimulated the uptake of AIB, glycine, L-leucine, L-lysine and L-arginine by rat kidney cortex slices. They showed that the stimulation of amino acid accumulation was sodium-dependent and that there was a delay in stimulation of amino acid transport of approximately two hours. The authors further showed that dibutyryl cAMP only stimulated the sodium-dependent component of L-lysine accumulation. They did not explain the enhancement of leucine accumulation by dibutyryl cAMP. Leucine accumulation has been found to be mediated solely by sodium-independent transport systems in most tissues. A similar system has been described for amino acid accumulation by fetal rat calvaria (86). Dibutyryl cAMP only enhanced the accumulation of amino acids that are transported by sodium-dependent systems. The authors also reported that the response to dibutyryl cAMP was delayed for two hours. Both groups found that, in agreement with our results, dibutyryl cAMP was more effective in enhancing amino acid accumulation than was cAMP. Many authors have postulated that dibutyryl cAMP enters cells more rapidly than cAMP (87), but it has been shown that cAMP enters cells grown in tissue culture more rapidly than its dibutyryl derivative (88). Recent studies have shown that dibutyryl cAMP inhibits the phosphodiesterase that converts cAMP to 5'-AMP and thereby facilitates the intracellular accumulation of endogenously produced cAMP

(89). This would account for the greater effectiveness of the dibutyryl derivative of cAMP. It has also been shown that dibutyryl cAMP is more effective than cAMP in stimulating chloride transport across both the amphibian (67) and rabbit cornea (68). The increase in chloride transport described by these authors was immediate and transient while the effect of these agents on amino acid accumulation is delayed. It therefore appears that this stimulation of chloride transport is not responsible for the increased amino acid accumulation and that these two responses do not have any cause and effect relationship. The stimulation of amino acid accumulation by dibutyryl cAMP does not appear to have physiologic significance because concentrations less than 1 mM do not cause a significant increase in amino acid accumulation. We further suggest that the increase in chloride transport is not physiologically significant for the same reason.

Various compounds which either mimic or potentiate the action of acetylcholine are used in the treatment of glaucoma. These drugs fall into four categories: irreversible cholinesterase inhibitors (echothiophate iodide, diisopropylfluorophosphate), reversible inhibitors of cholinesterase (eserine), parasympathomimetic alkaloids (pilocarpine), and derivatives of acetylcholine (Mecholyl)(90). All these drugs relieve the symptoms of glaucoma by increasing the true outflow facility of the aqueous humor through

the trabecular meshwork of the Canal of Schlemm. The cholinesterase inhibitors potentiate the action of acetylcholine at the postsynaptic membrane by binding with the cholinesterase molecule so that it is ineffective in hydrolyzing acetylcholine. It has been reported that some patients develop cataracts after treatment with the organophosphorous anticholinesterase agents echothiophate iodide (Phospholine iodide) and diisopropylfluorophosphate. Because all agents that are applied to the eye come into contact with the corneal epithelium we studied the effects of Phospholine iodide upon AIB accumulation by the cornea. The finding that Phospholine iodide does inhibit AIB accumulation may be clinically important because the effect was relatively specific for amino acid transport, as shown by the inability of Phospholine iodide to inhibit sugar transport. The lack of effect of eserine is consistent with the fact that there have been no reports of eserine induced cataracts. Mecholyl, an analog of acetylcholine, was also without effect but this compound has a low lipid solubility and therefore penetrates the cornea slowly.

C. Ion Transport in the Toad Cornea

Studies on ion transport in the cornea of the frog, *Rana catesbeiana* have shown that a potential difference is maintained across this tissue with the tear or epithelial surface negative with respect to the aqueous humor (22).

This potential difference is attributed to a net transport of chloride from the endothelium to the epithelium (22) and a smaller transport of sodium in the opposite direction (23). This net transport of chloride accounts for 80-90% of the short-circuit current of the in vitro frog cornea. Our studies have shown that the toad cornea also develops a potential difference with the epithelial side negative with respect to the endothelium. But we have also found that only 58% of the short-circuit current is due to the net transport of chloride from the endothelium to the epithelium, the remaining 42% of the short-circuit current being accounted for by a net transport of sodium from the epithelium to the endothelium. The increase in short-circuit current and potential difference caused by ascorbic acid is primarily due to a four-fold increase in the net flux of chloride from the endothelium to the epithelium. The question arises as to whether this increase in the net flux of chloride is due to a specific action on a chloride-specific transport enzyme or a more generalized increase in the permeability of the cornea. The fact that the effect of ascorbate is rather specific for the chloride ion, and that it is not associated with a change in the extracellular space or wet weight of the cornea, indicates that ascorbate is affecting a specific component associated with chloride transport. This argument for a specific effect of ascorbate receives further support from our amino

acid studies in which we find that sodium-dependent transport processes are stimulated. A possible mechanism for this ascorbate stimulation of transport processes was discussed previously. In the following section we would like to discuss this hypothesis in relation to ascorbate stimulated ion transport.

With regard to amino acid transport, we have presented evidence that ascorbate is acting as an electron donor to a microsomal electron transport system and our studies on ion transport provide further support for this theory. The inability of rotenone, dinitrophenol, and cyanide to block the increase in short-circuit current and potential difference caused by ascorbate provide support for this theory. The lack of effect of ATP provides further support for this theory. This is not the first time that an extra-mitochondrial electron transport chain has been implicated in ion transport. Kidder, Curran and Rehm have examined the possibility that such a system may be responsible for hydrogen ion secretion in the bullfrog gastric mucosa (91). They have found that changes in the rate of hydrogen ion secretion are closely correlated with changes in the oxidation-reduction state of cytochrome c. Acid secretion caused cytochrome c to shift to a more reduced steady state. The relative amounts of cytochrome b and c in this tissue were also determined and it was found that the amount of cytochrome c greatly exceeded that of cyto-

chrome b. This lead these authors to postulate that extra-mitochondrial cytochrome c might be specifically involved in the transfer of hydrogen ions. In regard to the cornea, studies of calf corneal epithelium have indicated the participation of ascorbic acid and glutathione as hydrogen carriers in an electron transport system involving substrates oxidizable by NADP and linked to the hexosemonophosphate shunt (92). The physiologic role of this electron transport system, as the authors saw it, was the reoxidation of NADPH produced by the hexosemonophosphate shunt which is very active in the corneal epithelium (93).

D. Summary

In conclusion, we have shown that neutral amino acids enter the cornea of the toad, Bufo marinus, by at least three separate transport mechanisms. The amino acids enter across the endothelial surface from the aqueous humor. It is not clear that the active step in amino acid accumulation occurs in the single layer of endothelial cells. In fact, the time-dependent nature of the inhibition of uptake caused by removal of sodium indicates that the active step occurs in a more remote area of the cornea. We have further shown that physiologic concentrations of lactate and ascorbate stimulate both the transport of amino acids (by a system closely resembling the L system) and chloride. These agents appear to be acting by reducing a specific

transport related protein. Amino acids are required by the cornea for protein synthesis. Active ion transport has been related to the maintenance of normal corneal transparency and hydration. It appears that reducing agents, found in high concentrations in the aqueous humor, play a significant role in the transport properties of the cornea and may be one of the mechanisms by which the avascular cornea is provided with energy. We have further shown that lactate is more effective in stimulating amino acid transport while ascorbate is a more effective stimulant for ion transport. This indicates that the transport system for amino acids is not the same as the transport system for chloride and that they are linked to the electron transfer chain at different sites.

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